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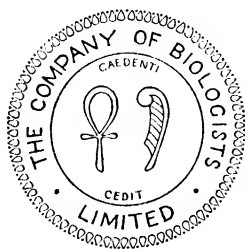
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The Influence of a Gonadotrophin on the Seasonal Changes in the Testis and Deferent Duct of the Chaffinch (*Fringilla coelebs*)

BY

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(From the Dept. of Endocrinology, Zoological Laboratory, University of Utrecht)

With 3 Plates

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INTRODUCTION

TO investigate the localization of the endocrine function of the testis two methods may be applied:

- (a) activation of the immature gonad by administration of gonadotrophins in juvenile animals;
- (b) activation of the quiescent gonad by administration of gonadotrophins in adult animals possessing a sexual cycle.

In a recent investigation (Sluiter and van Oordt, 1947) the first method was applied and domestic cockerels were used. In this paper it was proved that the classical Leydig cells of the intertubular testis-tissue, called lipoid cells by us, do not secrete the male sex hormone, but that this hormone is produced by the so-called secretion cells, also present in the intertubular tissue.

Then the question arose whether this also applies to adult wild birds with a distinct sexual cycle; so we used the second method mentioned above: adult male chaffinches were treated in different seasons with a gonadotrophin, and the histological and cytological changes induced by this hormone were studied. Just as in the experiments with cockerels gestyl was administered, a gonadotrophin prepared from pregnant mare serum. Contrary to its effect in cockerels it has a definite influence on the generative testis-tissue in

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(1)

chaffinches; in this paper therefore the hormonal influence of gestyl on both intertubular tissue and testis-tubules will be considered.

MATERIAL AND METHOD

The material consisted of 11 adult male chaffinches (*Fringilla coelebs*), which had been caught at Loosduinen near The Hague during their autumn migration. They were kept in a large aviary, placed near the window of one of the laboratory rooms. Here they showed all characteristics of the natural sexual cycle. Five birds received gestyl injections in different months of the year. Each experimental bird received 5 doses of 100 I.U. every other day, i.e. 500 I.U. in total, and was autopsied 2 days after the last injection. Two of the experimental birds were killed on 30 November 1943, one on 28 January 1944, one on 5 May 1944, and one on 11 August 1944. The testes were measured immediately *post mortem*. Parts of the same testis were fixed in formalin for general staining with haemalum and eosin or for staining with Sudan III to demonstrate lipoids and with Schultz's fluid to establish the presence of cholesterol. Others were fixed in Champy's fluid for staining cytological details such as mitochondria and granules with the aid of Altmann's acid fuchsin and brilliant-cresylblue. The thickness of the sections was 2–3 μ after Champy-fixation and 7–12 μ in all other cases. In addition, a part of about 3 mm. length was cut out of each left deferent duct at the level of the posterior end of the left kidney, fixed in formalin for general staining, and cut into sections, 7 μ thick.

RESULTS OF GESTYL-ADMINISTRATION

1. In the Quiescent Stage

As in other passerine birds the testes of the chaffinch already show a distinct regression just after the breeding season: then they decrease enormously in size, whereas the sperm-cells and spermatogenous cells degenerate for the greater part. In August a complete resting stage is reached, which passes over into the progressive stage towards the end of January.

In this paragraph the results will be described, obtained from 4 control birds and from 3 experimental birds, whose testes were quiescent at the beginning of the experiment. When compared among themselves neither the testes of the control birds nor those of the experimentals showed essential differences.

The macroscopical effect of the hormonal injection on the testis size was very distinct. For convenience' sake we shall indicate the testis size only by one figure: the average size in mm. of the long and short axes of both testes. In control birds the size of the testis in the quiescent stage is about 1 mm.; in birds treated with gestyl in the same stage the testes are much larger and have a size of 2.25–4.25 mm. Consequently the question arises which part of the testis is responsible for this enlargement. Microscopical investigation of the testes showed the following details:

(a) *Testis-tubules.* The testis-tubules of *control birds* have a markedly small diameter, and mostly a minute lumen; they contain, in cross-sections, one or two basal rows of nuclei (Pl. I a). Most nuclei are small and oblong; the others are large, round, and inflated, and are surrounded by a distinct, round cell-body; they represent typical gonocytes. The other cells possess indistinct cell-contours. One gets the impression that these cells have a long cylindrical shape; their apices reach the centre of the testis-tubule, whereas their nuclei lie at their bases.

In preparations stained with Sudan III the central part of the testis-tubules is filled with large lipoid globules (Pl. I b). Moreover, Champy-preparations stained with acid fuchsin show that the cylindrical cells, mentioned above, are filled with vacuoles, surrounded by numerous mitochondria (Pl. I c). The contents of these vacuoles have obviously been dissolved in Canada-balsam, for in fresh preparations they are visible as an osmiophil black substance, identical with the sudanophil, orange-coloured lipoids (cf. Pl. I b). Consequently these lipoids must be stored intracellularly, a fact already mentioned by Loisel (1903) for the winter testis of the sparrow.

We are inclined to suppose that these lipoids are the last remains of the fatty degeneration of the previous generation of spermatogenic cells; they must first have been present in the lumen of the testis-tubules, from which they have been resorbed later on by the cylindrical cells, mentioned above.

According to Rowan (1929) in juncos (*Junco hyemalis*) the gonocytes with their large inflated nuclei are destined to degenerate before long. Loisel (1901) is of opinion that in sparrows the cells with small nuclei are the cells from which the next spermatogenesis starts. According to Rowan (1929) and Bullough (1942) the same holds good for the junco and the starling respectively. As we have investigated only a small number of chaffinches, we could not decide this question.

In the *experimental birds* the testis-tubules possess a quite different structure. Their diameter is much larger, which is the principal cause of the considerable testis-enlargement (cf. p. 2 for measurements). This increase in size is accomplished partly by the formation of a lumen in the tubules, partly by a prominent thickening of their walls. As Pl. I a₁ shows, the latter phenomenon must not primarily be ascribed to cell-divisions but to a noteworthy inflation of the cell-protoplasm. It is true, cell-divisions have taken place, but only for the purpose of maintaining a single row of nuclei at the periphery of the tubules.

Contrary to the nuclei in the testis-tubules of the control birds, the nuclei in the tubules of the experimental birds have all become round and inflated; therefore a distinction between gonocytes and cells with small nuclei is impossible here. Meiotic figures (Pl. I a₁) are very scarce or lacking.

The cell-plasma possesses a foamy structure and contains many large vacuoles. After Sudan III staining several lipoid-globules are visible (Pl. I b₁). In Champy-preparations of the experimental testes (Pl. I c₁), which

are quite different from those of control testes (cf. Pl. I c), the mitochondria have spread and the compact accumulation of lipid vacuoles does not exist any longer.

From the above it follows that the testis-tubules of chaffinches, treated with gestyl during the quiescent stage, increase distinctly in diameter. The histological structure of these tubules, however, does not show any likeness to that of testes with a beginning spermatogenesis. As the number of control birds is small, we are not sure whether this structure occurs in normal chaffinches or not, but this seems improbable, as it has not been observed in related bird-species, viz. sparrows (Loisel, 1901, 1903), juncos (Rowan, 1929), starlings (Bullough, 1942), and greenfinches (Damsté, 1947).

(b) *Intertubular Tissue and Deferent Ducts.* The histological changes in the intertubular tissue, brought about after gestyl-administration in chaffinches during the quiescent stage, are still more conspicuous.

In the chaffinch, as well as in other birds, the intertubular tissue of the *quiescent testis* is composed almost exclusively of packed masses of small oval nuclei, mostly called connective tissue nuclei in other bird-species (Benoit, 1923a; Rowan, 1929; Bullough, 1942; Damsté, 1947). The testis-tubules are enveloped by these masses, which are separated from each other by a narrow layer of connective tissue cells and blood-vessels (Pl. I c and I d). Though most of the cells belonging to the masses surrounding the testis-tubules probably pass over into differentiated connective tissue cells, we prefer to call them 'indifferent cells' in this stage of development, as then true differentiated connective tissue cells are also present.

Neither lipid (= Leydig) cells nor secretion cells can be found in the intertubular tissue of quiescent testes of control chaffinches. The fact that lipid cells are lacking follows also from the observation that the sudanophil globules (cf. Pl. I b) are small, diffusely spread in this tissue, and obviously lying in the indifferent cells.

In the testes of chaffinches, treated with gestyl during the resting stage, the intertubular tissue has a quite different structure. In the first place its quantity has increased (Pl. I d_1 ; Pl I d and d_1 are comparable, as the radius of each semicircle is proportionate to the average sizes of the long and short axes of both testes). Moreover, contrary to the intertubular tissue of the control testes, the intertubular tissue of the testes of the experimental birds consists of differentiated connective tissue cells, between which groups of true interstitial cells are found, lacking in control testes. Some of these cells are lipid cells, which follows from the concentration of the sudanophil elements (cf. Pl. I b_1), but for the greater part they are true secretion cells, characterized by many fuchsinophil elements and the lack of lipid vacuoles (cf. Pl. I c_1).

That a great quantity of androgenic substances is produced by this large number of secretion cells may be derived from the fact that the diameter of the deferent ducts of the experimental birds (Pl. I e_1) is much larger than that of the controls (Pl. I e): in the course of 10 days the very narrow

deferent duct of the quiescent bird nearly reached the normal diameter of the reproductive season (Pl. III *e*).

Finally we have to answer the question whether during the normal sexual cycle of the chaffinch an intertubular tissue of similar appearance develops.

After having studied the testes of our control birds, we came to the conclusion that such a large number of secretion cells is never found during the normal sexual cycle of the testis. Normally the increase in diameter of the deferent ducts from the quiescent winter-condition to the functional spring-condition takes more than 10 days, the period of increase in our experimental birds. So it is easily understood that under artificial conditions the abnormally quick increase in diameter of the deferent duct only takes place under the influence of an abnormally high level of male sex hormone, secreted by an excess of secretion cells.

From the above it follows that after gonadotrophin-administration not only an increase in the quantity of inter-tubular tissue is induced, but also its differentiation. Indifferent cells develop into differentiated connective tissue cells, and into special interstitial cells, of which lipid and secretion cells may be distinguished. As, moreover, the male genital ducts react, these secretion cells must have secreted androgen. We came to the same conclusion in our paper on the reaction of the intertubular tissue to gestyl in cockerels (Sluiter and van Oordt, 1947).

2. In the Progressive Stage

At the end of January the first changes in the testes of our control birds were visible. Therefore we chose this time of the year for gestyl-administration. Macroscopically the influence of the hormone on the testes was distinct: the testes measured 3 mm., the control testes only 1.5 mm. As to the size there was no great difference between the testes of January and of August or November control birds. Microscopical investigation, however, showed that the testes of the January control birds possessed a distinctly different structure.

(a) *Testis-tubules.* In the *control bird* the diameter of the testis-tubules, in which a lumen is scarcely visible, is still small; but the wall of these tubules contains several layers of nuclei of almost the same size (Pl. II *c*). Some of these nuclei are surrounded by distinctly limited round cell-bodies, and therefore strongly resemble gonocytes; most cells, however, form a syncytium, containing many minute, dispersed mitochondria. The lipid globules, so characteristic for the tubules of the resting stage, are much reduced in size and number (Pl. II *b*).

In chaffinches *injected with gestyl* in January, the testis-tubules on the whole show the same characteristics as testis-tubules of birds injected in August and November. Only the quantity of lipoids (Pl. II *b*₁) is smaller, which is, however, a matter of course, as the quantity of lipoids has also decreased in the testis-tubules of control birds.

Therefore testis-tubules of birds injected with gestyl in the beginning of

the progressive stage react in the same way as tubules of birds treated during the quiescent stage.

(b) *Intertubular Tissue and Deferent Ducts.* The histology of the intertubular tissue of a *control bird* of January (Pl. II *d*) is totally different from that of the control birds of August and November (Pl. I *d*). The main part of this tissue consists of differentiated connective tissue cells, whereas the indifferent cells only form a narrow layer around the testis-tubules. In the connective tissue groups of special interstitial cells are to be found. In these respects there is much resemblance to the intertubular testis-tissue of chaffinches treated with gestyl in August and November (Pl. I *d*₁). Consequently one should expect growth and differentiation of the deferent ducts in control birds killed in January. However, this is not the case (Pl. II *e*). Making use of Champy-preparations we found (Pl. II *c*) that the special interstitial cells (black in Pl. II *d*) are exclusively lipoid cells; these are also visible (Pl. II *b*) in the testis-section of the same control bird, where a concentration of sudanophil elements in definite parts of the interstitial tissue is distinct.

Secretion cells, which in our opinion are the essential source of the male sex hormone, have not yet been formed, which explains the slight development of the deferent duct (Pl. II *e*).

The size and histological structure of the intertubular tissue and of the deferent ducts of *gestyl-treated* January birds differ little from those of *gestyl-treated* August and November birds. In the January birds the number of lipoid cells is larger (Pl. II *b*₁) than in the others (Pl. I *b*₁); however, secretion cells are also present. Consequently the deferent ducts are abnormally well developed for the time of the year. The results of our experiments in August and November have therefore been confirmed by those carried out in January.

3. *In the Mature Stage*

In May chaffinches are completely mature sexually; an effect of *gestyl-injection* was not visible macroscopically: both experimental and control birds possessed testes of 6.5 mm. The histological differences were also small.

(a) *Testis-tubules.* Pl. III *d*, drawn from a testis-section of a sexually mature chaffinch, shows that the enlargement of the testes in the reproductive season is only the result of the enormous development of the testis-tubules. (When comparing one must take into account that in Pl. III *d* the radius of the semicircle is proportionate to *half* the average lengths of the axes of the testis.)

The histological structure of the testis-tubules is the same in experimental and in control birds: in both cases spermatogenesis is complete with many ripe sperms, their heads arranged in bundles on Sertoli cells (Pl. III *c*).

Consequently *gestyl-injection* in chaffinches in May does not have any influence on spermatogenesis.

(b) *Intertubular tissue and deferent ducts.* Contrary to the opinion of most of the earlier authors (e.g. Watson, 1919), who thought that the quantity of intertubular tissue in the ripe testis is much smaller than in the quiescent one, Benoit (1923*b*) holds the view—with which we fully agree—that this only seems to be so. Though the intertubular spaces have become very narrow, the total volume of the intertubular tissue need not necessarily have decreased. Moreover, Benoit points to the fact that the quantity of the special interstitial cells, not the size of the intertubular tissue, is of primary importance for the endocrine function of the testis.

From Pl. III *c* it follows that the intertubular tissue of the mature May-testis is histologically different from that of other seasons. Besides differentiated connective tissue cells and blood-vessels many lymph-spaces occur, lacking in the intertubular tissue of testes of other seasons. We cannot give any explanation of the physiological significance of these lymph-spaces; we will only state that they are also common in rodents (Sluiter, 1945). In addition, special interstitial cells are present in the intertubular tissue of the ripe testis (Pl. III *d*, black), their total number being about the same as after gestyl-administration in May. Most of them are lipoid cells which are still more numerous in the experimental (Pl. III *b*₁) than in the control birds (Pl. III *b*). Moreover, some secretory cells are present (Pl. III *c*). From this observation it is obvious that a small number of secretion cells suffices to produce the small quantity of sex-hormone needed to maintain the activated stage of the male genital ducts (Pl. III *e*).

Our observations clearly show that the effect of gestyl-injection on the testis in May is very slight or lacking; this is not surprising, as the mature testis-stage is normally also reached at this time of the year.

DISCUSSION

1. *Hormonal Regulation of Spermatogenesis*

In 1931 Schockaert and in 1935 Witschi and Keck demonstrated that in birds, spermatogenesis is normally caused by gonadotrophins of hypophyseal origin. With preparations derived from mammalian hypophyses complete spermatogenesis was induced in juvenile domestic ducks and in adult sparrows in the quiescent stage respectively.

As to investigations in which gonadotrophins, prepared from pregnant urine, were administered, the results do not agree. Schockaert (1933) got no results in cockerels and juvenile ducks when using such a preparation; Lahr, Riddle, and Bates (1941), however, observed a definite influence on the testes of pigeons and doves. Moreover, de Fremery (1941) induced complete spermatogenesis in sparrows in February, with the aid of pregnyl, a preparation from pregnant urine. Neither did investigations with the third type of gonadotrophins, i.e. those derived from pregnant mare serum, give corresponding results. The present authors have recently communicated that gestyl, a preparation from pregnant mare serum, has no influence on the

spermatogenous tubules of cockerels (Sluiter and van Oordt, 1947). Witschi and Keck (1935) had the same negative result after daily administration of 50 R.U. of another chorionic gonadotrophin in male sparrows, but de Fremery was able to induce 'a similar development of the testicles' (1941, p. 188) in sparrows with gestyl and pregnyl, i.e. he obtained complete spermatogenesis. This observation was confirmed by Pfeiffer and Kirschbaum (1943), also in sparrows, and by Lahr, Riddle, and Bates (1941) in pigeons and doves.

The present investigation shows distinct histological changes in the testis-tubules of chaffinches after gestyl administration; spermatogenesis, however, is not induced. Consequently our results do not harmonize with those of de Fremery, who used the same preparation and a related species. This difference in result may partially be explained by the quantity of hormone administered: de Fremery's sparrows got each 1,000 I.U. of gestyl in total, whereas our chaffinches received only half this amount. But this does not explain the fact that in the testes of our experimental birds no initial stages of spermatogenesis were induced; in our birds the diameter of the spermatogenous tubules only increased in size, due partly to the formation of a lumen, and partly to the plasma of the cells becoming distinctly inflated. The same histological structure is never found during normal spermatogenesis and therefore cannot be considered as an initial stage of spermatogenesis.

This observation clearly shows that after hormone injections neither the sizes nor the weights of the testes are indications of an active spermatogenetic stage.

2. *Function of the Intertubular Tissue of the Testis*

In the literature dealing with the interstitial cells—often called 'true Leydig cells'—of the testes of normal birds contradictory opinions about their function are met with. In some cases even their presence in one bird-species is denied by one author, whereas it is stated by others. For instance, Benoit (1936) found interstitial cells in the normal duck, in which they were not traced by Schockaert (1931), and in the sparrow they were described by Pellegrini (1925) but not observed by Pfeiffer and Kirschbaum (1943).

For these contradictions two causes may be put forward:

- (a) There is no agreement on the definition of the interstitial cells.
- (b) To demonstrate presence and function of the interstitial cells the ordinary laboratory routine techniques are insufficient.

As most of the investigators of mammalian or avian testes were not aware of the second cause or did not agree with it (Pfeiffer and Kirschbaum, 1943, p. 219), we think it superfluous to compare their results with ours.

The normal cytology of the interstitial cells of the testes of an exotic bird-species with a distinct sexual cycle, i.e. in the 'combassou' (*Vidua* sp.) has been described by Benoit (1923a). His technique, the only one suitable to

study these cells, was the same as ours: fixation in an osmic liquid and staining according to Altmann's technique. He stated that in the beginning of the progressive stage of the testis a number of cells in the intertubular tissue assume a glandular appearance, which process accentuates as the spermatogenesis makes progress. When the testis-tubules have attained their maximum size, cells with glandular structure, secreting the male sex hormone, are still present in the intertubular spaces, according to Benoit.

Only in one point do Benoit's and our conclusions differ essentially: Benoit does not make a sharp distinction between lipid cells (i.e. cells of Leydig) and secretion cells, which he takes together as 'tissue glandulaire', whereas the present authors have demonstrated (Sluiter and van Oordt, 1947) that in the testis of the domestic fowl the sex hormone is only produced by the secretion cells. Our results in chaffinches, described above, are in full agreement herewith (cf. p. 6).

Another question is, whether the lipid cells can pass over into secretion cells, as Benoit (1929) thinks. According to us the following points are in favour of this opinion:

1. Notwithstanding the fact that in all birds so far investigated the lipid cells possess a very high content of lipoids, the structure of their nucleus, their size, and their large quantity of fuchsinophil elements are very similar to those of secretion cells.
2. The lipid, which is present in these cells in avian and mammalian testes, consists, at least partly, of cholesterol; in the present investigation this also was found by us by the aid of Schultz's cholesterol test. As cholesterol is the substance from which the synthetic androgens are prepared, it is not unthinkable that the lipid cells are destined to pass over into hormone-secreting cells. However, in our opinion this is by no means proved.

Against it the following facts can be raised:

1. In Stieve's fattening experiments (1923, 1926) the quantity of lipid cells in the testes of geese and cocks was increased considerably, but an increased hormone-production could not be established.
2. It was found by many authors that the testes of the animals investigated did not contain 'classical Leydig cells' (i.e. our lipid cells), whereas male sex-hormones were formed in these organs.

From the above we may conclude that, as yet, lipid and secretion cells must be distinguished from each other as two cell types with different functions. As to the lipid cells, it is certain that their presence in the intertubular testis-tissue is a reflection of the trophic role of this tissue, supporting either the endocrine or the generative or both testis-functions. In chaffinches the trophic activity of the intertubular tissue is obviously subject to the seasonal periodicity.

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SUMMARY

1. Male chaffinches (*Fringilla coelebs*) were treated with gestyl, a gonadotrophin prepared from pregnant mare serum, in different seasons; using different techniques their testes and deferent ducts were histologically studied after autopsy on 11 August, 30 November, 28 January, and 5 May.

2. After Champy-fixation and Altmann-staining two types of interstitial cells can be distinguished in the intertubular tissue of sexually active chaffinches: lipid cells (= Leydig cells) and secretion cells.

3. Results of gestyl-administration: In summer- and winter-birds (Pl. I) whose testes are in the resting stage, the testis-tubule diameter shows a strong enlargement, which is partly due to the plasma of the cells being distinctly inflated; spermatogenesis does not take place. In the intertubular tissue lipid and secretion cells appear in abnormally large numbers. In 10 days the deferent ducts pass over from the quiescent into the fully activated stage. In spring-birds (Pl. III), being in the reproductive stage, the administration of gestyl has practically no effect. In this stage the intertubular tissue also contains both lipid and secretion cells.

4. From the results mentioned under 3, and the fact that in the control bird of 28 January, being in the beginning of the progressive stage (Pl. II), many lipid cells were found, whereas its deferent ducts were still quiescent, it is concluded that only the secretion cells produce the male sex-hormone. The lipid cells, which amongst others contain cholesterol, possess only a trophic function.

5. The difference in reaction of the seminiferous tubules of birds to chorionic and hypophyseal gonadotrophins as well as the function of the interstitial cells are discussed. Most opinions on the last-mentioned subject are not sufficiently well founded, as the investigators used routine techniques only for the cytological investigation of the interstitial tissue.

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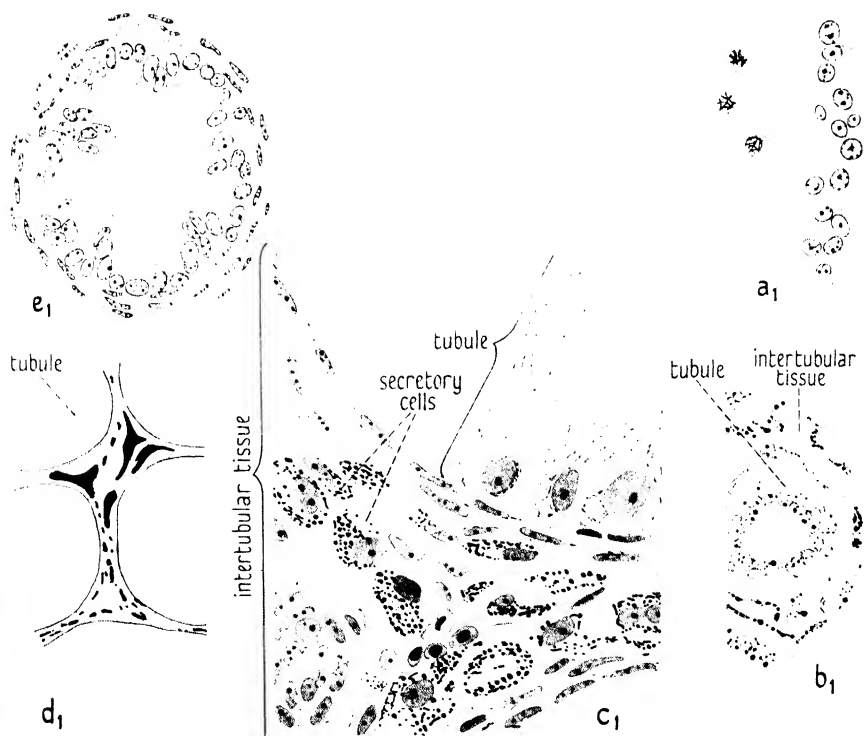
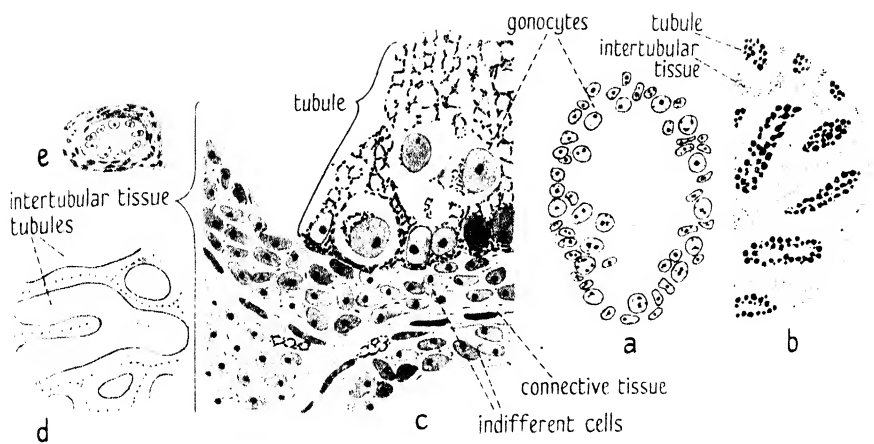
DESCRIPTION OF PLATES

PLATE I. Testis- and deferent duct-sections of chaffinches, killed in August and at the end of November. *a* and *a*₁, sections of testis-tubules stained with haemalum-eosin, respectively before and after gestyl-treatment ($\times 500$); *C* and *C*₁, testis-sections after Sudan III-staining, respectively before and after gestyl-treatment ($\times 100$); *c* and *c*₁, the same after Champy-fixation and Altmann-staining, respectively before and after gestyl-treatment ($\times 1,000$); *d* and *d*₁, the same (schematic, $\times 100$; testis-tubules white, indifferent interstitial cells grey, connective tissue dotted, differentiated interstitial cells black); *e* and *e*₁, sections of deferent ducts, stained with haemalum-eosin before and after gestyl-treatment ($\times 500$).

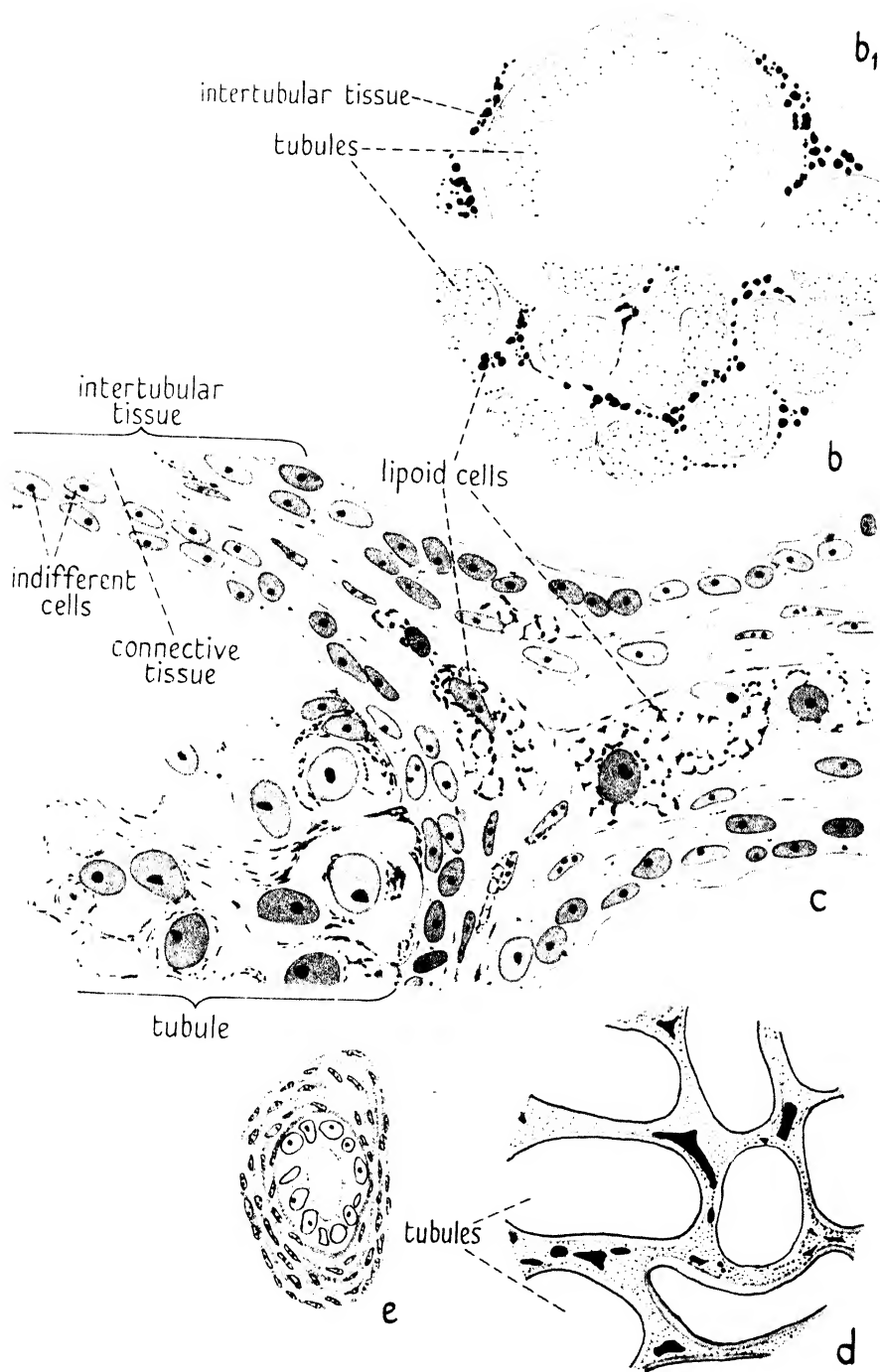
PLATE II. Testis- and deferent duct-sections of chaffinches killed at the end of January. *b* and *b*₁, testis-sections after Sudan III-staining, respectively before and after gestyl-treatment ($\times 200$); *c*, section of control-testis after Champy-fixation and Altmann-staining ($\times 2,000$); *d*, the same (schematic, $\times 200$; for white, grey, dotted, and black areas cf. Pl. I *d* and *d*₁); *e*, section of deferent duct of the same control bird after haemalum-eosin staining ($\times 1,000$).

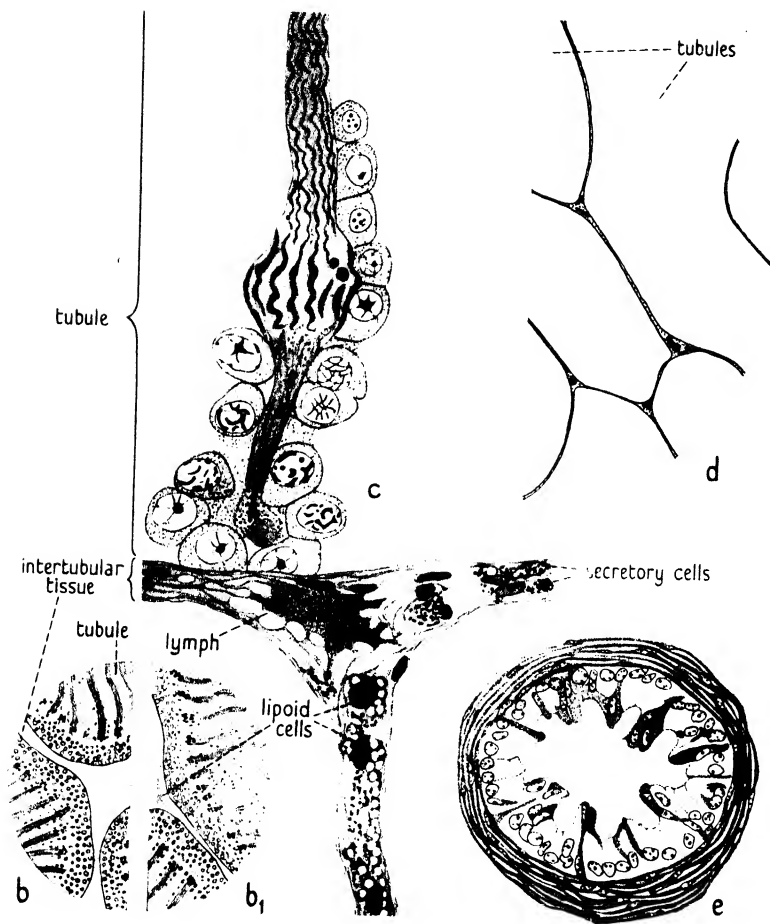
PLATE III. Testis- and deferent duct-sections of chaffinches killed at the beginning of May. *b* and *b*₁, testis-sections after Sudan III-staining, respectively before and after gestyl-treatment ($\times 100$); *c*, section of gestyl-treated testis after Champy-fixation and Altmann-staining ($\times 1,000$); *d*, the same (schematic, $\times 50$; for white, dotted, and black areas cf. Pl. I *d* and *d*₁); *e*, section of deferent duct of a control bird, stained with haemalum-eosin ($\times 500$).





J. W. SLUTTER AND G. J. VAN OORDT—PLATE I





J. W. SLUTTER AND G. J. VAN OORDT—PLATE III



The Structure of Whale Blubber, and a Discussion of its Thermal Properties

BY

D. A. PARRY

(From the Department of Zoology, University of Cambridge)

With one Plate

AS a warm-blooded animal, a whale must be adapted not only to prevent an excessive loss of heat but also to control the rate of heat loss in relation to changes in its metabolic activity and in the temperature of its surroundings. This paper contains an account of whale blubber considered from this point of view. The first part is devoted to the morphology of blubber, particular attention being paid to the blood circulation which does not appear to have been previously described; and the second part contains a discussion of the efficacy of blubber as a thermal insulator of controllable conductance. The results are based on a study of the Common Porpoise (*Phocaena phocaena*), and the Blue and Fin Whales (*Balaenoptera musculus* and *B. physalus*).

The term 'blubber' is in popular and commercial use to denote the superficial tissues of whales and seals, which form a compact layer loosely fastened to the underlying muscle and easily stripped off for commercial purposes. Blubber comprises the animal's epidermis, dermis, and hypodermal tissues; and in the following account the word will always be used in this sense. The term 'integument' is used to refer to these tissues in other mammals.

GROSS MORPHOLOGY

Area

The surface area of whales and porpoises was found by measuring the circumference or semi-circumference at intervals down the body and finding the area contained by the smooth curve drawn through these values plotted against length. Flukes, dorsal fin, and flippers (measured for the porpoise only) were traced on squared paper.

Phocaena phocaena. One male, 155 cm. long, was measured, the circumference being taken at intervals of 15 cm. The results were:

Area of body only . . .	9,950 cm. ²
„ flukes . . .	820 „
„ fin . . .	250 „
„ flippers . . .	540 „
Total area . . .	11,560 „

Assuming that the surface area is proportional to the square of the length, that is to say, $A = Kl^2$, we have:

$$K = 0.45 \text{ for the total area,}$$

$$K_1 = 0.39 \text{ for the body area alone.}$$

The value for K is in good agreement with that calculated from Gray's data (Gray, 1936) which gave $K = 0.44$.

Balaenoptera physalus. Measurements were made of one adult whale and several foetuses, the results being as follows. The figures refer to the body area alone, excluding fin, flukes, and flippers.

Length	Measurement interval	Area	K_1
19.8 m.	3 m.	137 m. ²	0.35
308 cm.	25 cm.	32,330 cm. ²	0.34
245 cm.	„	20,280 cm. ²	0.34
240 cm.	„	20,710 cm. ²	0.36

These figures give a mean value for K_1 of 0.35. This compares favourably with the value of $K_1 = 0.37$ derived from Laurie's data (1933) which were based on the assumption that a whale can be regarded as two cones fastened together at their bases.

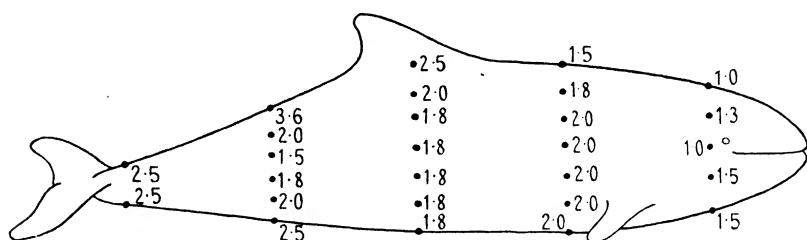
Thickness

Phocaena phocaena. The blubber thickness of a 155-cm. male was found both by direct measurement and indirectly from the area, weight, and density. The results of direct measurement are shown in Text-fig. 1 and give a mean thickness of 1.8 cm. The same figure was obtained by the other method, the density having been determined as 0.98 gr./c.c.

Balaenoptera physalus. It is difficult to study the distribution of blubber thickness under the conditions on board a factory ship, but from personal observations and those kindly made by Dr. M. Begg (Biologist, Factory Ship *Balaena*, 1946-7) I found that the thickness on the flank, level with the dorsal fin (the standard measuring-point of the Discovery Investigations), was approximately the mean value for the whole surface. It is now possible to amplify this conclusion as I have the advantage of a personal communication from Dr. E. J. Slijper embodying the results of extensive measurements made during 1946-7 and 1947-8. From these measurements I have calculated the mean thickness in two ways. Firstly, the outline of a whale was drawn on graph-paper so that it was divided into about eighty squares. Blubber thicknesses were marked in or interpolated, and the mean found from the sum of the product of areas and thicknesses. This gave a mean of 1.1 times the thickness at the standard place. The method may be suspected of error owing to the foreshortening of the dorsal and ventral surfaces, but as both the thickest and thinnest blubber is to be found on these surfaces (dorsal and ventral surfaces of the tail, and ventral surface of the throat and chest, respectively), the

resultant error is unlikely to be great. This contention is supported by the fact that the second method of calculation gave the same result as the first. This method was to find the mean of an equal number of thicknesses measured or interpolated along the dorsal, lateral, and ventral lines, and effectively allots equal areas to the dorsal, ventral, and lateral surfaces.

Extensive data are available concerning blubber thickness at the standard place. It varies with locality, time of year, and size of whale. Mackintosh and



TEXT-FIG. 1. *Phocaena phocaena*. Depth of blubber in centimetres.
Length of animal: 155 cm.

Wheeler (1929) give measurements made at South Georgia, while the following are taken from Dr. Slijper's communication and come from whales caught on the Antarctic pelagic whaling grounds:

	<i>Dec.</i>	<i>Jan.</i>	<i>Feb.</i>	<i>March</i>
	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>
Blue whales, ♂ 74'-80' .	11.7	10.6	12.6	12.1
" " ♀ over 80' .	15.2	14.0	16.0	16.4
Fin whales, ♀ 65'-70' .	7.8	9.6	11.2	12.7

MICRO-ANATOMY AND HISTOLOGY

Phocaena phocaena

Material

The following account is based on the blubber of a 155-cm. male porpoise which after death had spent 2-3 days in transit in Great Britain at air temperature (October) and a further 4 days in a cold room (0° C.) while a plaster cast was made, during which process local warming of the blubber must have occurred. Despite this treatment a considerable amount of cytological detail was preserved, as will be seen from the photomicrographs. The animal fortunately died with its peripheral blood-vessels dilated, and the blood remaining in the vessels enabled their course to be made out.

Methods

After the post-mortem delay described above, small pieces of material were fixed in Susa or in 5 per cent. formalin, both fixatives giving good results. For preparations of the epidermis, paraffin embedding was satisfactory and sections down to 4 μ were obtained; but this method was unsuitable for the

dermis and hypodermis as it hardened the connective tissue and dissolved away the fat so that the tissues were badly torn and distorted during cutting. Lendrum's method of softening with phenol (Carleton and Leach, 1938, p. 39) was tried unsuccessfully, and frozen sections were finally resorted to. Good preparations down to 20μ were obtained, and little difficulty was experienced in making up complete series by transferring each section to a slide treated with gelatin, and exposing the full slide to formalin vapour (Pantin, 1946, p. 26).

The epidermis was well stained by Heidenhain's haematoxylin. More difficulty was experienced with the other tissues, where a stain was required which would not obscure the red blood in the vessels and would show up the vessel walls so that arteries could be distinguished from veins. Mallory's triple stain, and Masson's trichrome stain, gave unsatisfactory results, and the required contrast between blood and tissues was finally obtained by staining lightly with 1 per cent. water-soluble aniline blue.

Tissue Components

The blubber of the porpoise is composed (Pl. I, fig. 1), like the integument of other mammals, of epidermis, dermis, and hypodermal tissue. Its most distinctive features are the thickness and almost exclusively fatty nature of the hypodermis, and the absence of hair, skin glands, and sense organs. The following account deals separately with the three tissue layers, and then considers the blood-supply. No sensory or vasomotor nerves have been found.

Epidermis

(i) *Micro-anatomy*. The thickness of this, the most superficial layer, varies little over the body, being 3.25 mm. or $\times(2 \times 10^{-3})$ of the body length. It varies in colour from white to dark grey, being responsible for the colour of the animal. The deeper half of the epidermis is penetrated by upward extensions of the dermis in the form of 'dermal ridges' running parallel to the long axis of the body, from the summits of which arise the dermal papillae. The section illustrated in Pl. I (fig. 1) is cut at right angles to the long axis of the body so that the ridges are cut transversely; the second ridge from the right-hand side has been cut between two papillae while all the others have papillae rising from them so that the distinction between the two is not evident. Pl. I, fig. 3 shows a transverse section through the ridges, and fig. 2, cut more superficially, shows the papillae which have arisen from the ridges. This arrangement differs from the condition found in most mammals where the papillae arise directly from the base of the epidermis.

There are approximately 25 papillae per sq. mm., which is about half the mean density found in man (Lewis, 1927, p. 253).

(ii) *Histology*. The epidermis is composed of two layers, the superficial *stratum corneum* (unstained in Pl. I, fig. 1); and a deeper layer which on comparative grounds will be called the *stratum germinativum*, although no mitoses have been observed. The transition between the two layers is characterized by a

flattening of the cells parallel to the surface, a thickening of the cell walls or inter-cellular substance, and a reduction of nuclei and cytoplasm. There is no *stratum granulosum* or *stratum lucidum*, a condition not unusual in other mammals.

As in other mammals the *stratum germinativum* may be subdivided into 'cylindrical cells' with large nuclei, lining the dermal ridges and papillae; and polygonal 'prickle cells' with well-developed intercellular and intra-cellular fibrils, lying superficial to the papillae.

In the pigmented parts of the blubber, pigment granules occur in the epidermis; most densely in the cylindrical cells of the *stratum germinativum*, especially those immediately bordering the ridges and papillae; and less densely in the prickle cells. Pl. I, figs. 2-4 show the granules in unstained sections.

Dermis

This consists of a mat of connective tissue fibres parallel to the surface, very dense at the base of the epidermis (Pl. I, fig. 1), and becoming less dense and invaded by fat cells at deeper levels where it merges into the underlying hypodermis. Its thickness is about 0.34 mm., or $\times (0.22 \times 10^{-3})$ of the body length. This tissue is formed predominantly of white fibres, staining characteristically with v. Giesson's stain. No elements took up orcein, but a few elastic fibres were recognized in some preparations.

Hypodermis (note: only a fraction of the total thickness of the tissue is shown in Pl. I, fig. 1)

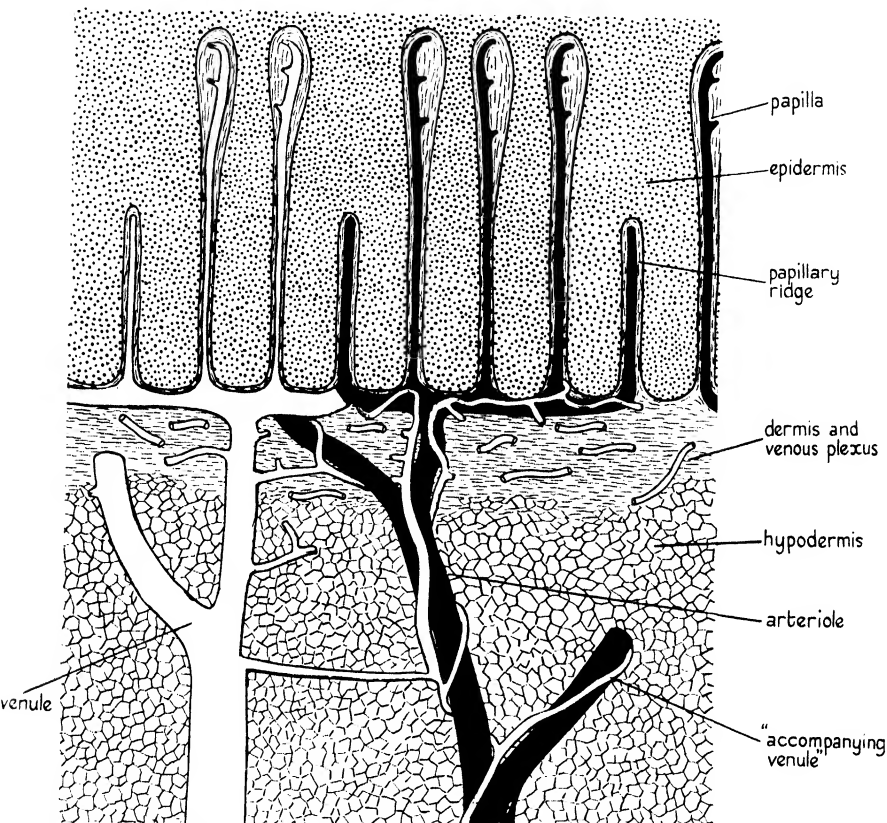
This tissue is composed almost entirely of fat cells, with occasional bundles of white fibres running irregularly among them (Pl. I, fig. 1). The great development of this layer is responsible for the thickness of blubber as a whole, and for its commercial value. It corresponds to the panniculus adiposus of that other almost hairless mammal, man; and presumably also to the thick layer of fat beneath the skin of the domestic pig.

Blood-supply (see Text-fig. 2, which has been constructed from a large number of serial drawings)

(i) *Arteries*. Arterioles run up through the hypodermis, branching as they do so; and through the dermis to the base of the epidermis. Here they run somewhat obliquely across the bases of the dermal ridges, giving off twigs which run up the ridges to join the capillaries in the papillae. The most careful examination has failed to show the arterioles giving rise to capillaries or forming a plexus in the hypodermis or dermis, neither are there any signs of arteriovenous anastomoses. The only course open to the blood appears to be up the twigs in the ridges to the capillaries in the papillae. Text-fig. 3 (a) shows an arteriole running through the dermis to the base of the epidermis. Pl. I, fig. 3 shows both arterial and venous twigs in the dermal ridges, running upwards, and also horizontally to supply the papillae arising from the

summits of the ridges. It is probable that even at this level, arterial and venous vessels are distinguished by the thickness of their walls.

(ii) *Capillaries*. Capillaries occur in the papillae (Pl. I, fig. 2) where the terminal twigs are probably joined together by several short capillaries, as



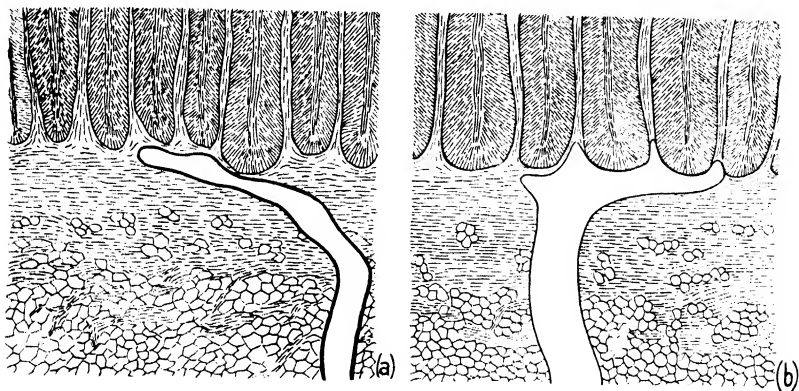
TEXT-FIG. 2. *Phocaena phocaena*. Blood circulation in the superficial regions of the blubber. To avoid confusion, arterial and venous vessels are not both shown in the same dermal ridge and papilla. Based on numerous serial drawings.

photomicrographs of living tissue have shown to be the case in man (see Lewis, 1927, p. 13). The diameter of the capillaries is about 15μ .

(iii) *Veins*. Venous twigs running down the dermal ridges are collected into venules which, like the corresponding arterioles, run obliquely across the base of the epidermis and then down through the dermis and hypodermis, joined by venules from adjacent parts of the skin. Text-fig. 3 (b) shows a vertical section through the dermis and base of the epidermis, with one of these venules running horizontally just below the epidermis collecting twigs from the ridges, and then turning to run down through the dermis. Pl. I, fig. 4, is a thick,

horizontal section through the extreme base of the epidermis, showing a collecting venule running just below it.

Unlike the corresponding arterioles, these venules are associated with an extensive plexus in the dermis and the transitional region between dermis and hypodermis. This plexus is shown in Pl. I, fig. 5. It appears to be a single network, with elements running in both the horizontal and vertical planes (see Pl. I, fig. 1), and so the short lengths of plexus shown in Text-fig. 2 must be imagined as inter-connected in planes above and below that of the drawing. This text-figure illustrates the fact that the venules preserve their identity



TEXT-FIG. 3. Vertical sections through the dermis and base of the epidermis showing (a) an arteriole, and (b) a venule, running up to supply the dermal ridges and papillae. Based on 100μ frozen sections, stained with aniline blue.

up to the base of the epidermis, connecting with the plexus by means of short branches; they do not divide up into the plexus which in turn gives rise to more superficial vessels. Thus blood from the papillae may run directly down through the dermis and hypodermis in the large venules; or it may flow through the narrow plexus vessels under the influence of small pressure differences between one part of the plexus and another. The diameter of the plexus vessels is about 30μ .

In addition to the large venules already described, which pursue an independent course through the blubber, there are much smaller venules, often not more than one, accompanying the arterioles in at least the superficial layers of the hypodermis, and in the dermis up to the base of the epidermis but not entering the dermal ridges (see Text-fig. 2). These vessels, like the large ones, contribute to the venous plexus, and are occasionally connected with the large ones by branches in the hypodermis.

Balaenoptera

The sample of the blubber of *Balaenoptera physalus* brought to England in a hard-frozen state for heat-conductivity measurements (see below) also

afforded material for histological examination. The peripheral blood-system could not be followed as it did not contain blood, but the micro-anatomy of the three tissue layers—epidermis, dermis, and hypodermis—was similar to that of *Phocaena* with the exception of a peculiar vacuolation in the epidermis which we suspect to be an artifact due to freezing (see next paragraph).

We have also examined a sample of the blubber of *Balaenoptera musculus* kindly supplied by Dr. Michael Begg, who fixed it in formalin shortly after the capture of the whale. The micro-anatomy of the tissue layers was also similar to that of *Phocaena*, and the vacuolation noted above did not occur. The blood-system has not been traced out in detail, but the main elements found in *Phocaena* were all present: arterioles running through hypodermis and dermis to give off twigs to the dermal ridges; collecting venules receiving twigs from the ridges and running down through dermis and hypodermis; and plexus in the dermis.

DISCUSSION OF THE MICRO-ANATOMY AND HISTOLOGY

A few previous accounts exist of the blubber tissues, excluding the vascular system. That of Bonin and Vladykov (1940) deals with the Beluga (*Delphinapterus leucas*) where the dermis is relatively better developed than in our species, so as to be of commercial importance in the manufacture of shoe-laces. These authors do not mention the dermal ridges; otherwise their account is in good agreement with ours. Stiglbauer (1913: *Delphinus delphis*) also does not describe the dermal ridges, nor does he find 'prickle cells' in the epidermis. Japha (1907: *Balaenoptera* spp.) gives a clear account of the ridges, from which Schumacher (in Bolk *et al.*, 1931) has made a good perspective drawing (Fig. 248, p. 468). In other respects, too, we are in general agreement with Japha, and his account of five different layers in the epidermis, as compared with our three, seems to be a refinement of description rather than a difference of substance.

We know of no other account of the blubber vascular system. This differs from that in the integument of man (Spanholtz, in Lewis, 1927) where the plexuses are much more complicated: the arterioles break up into two separate ones at different levels, while the venules divide up into no less than four. The 'accompanying venules', which we have described as running beside the arterioles, do not occur in man.

THE THERMAL PROPERTIES AND FUNCTION OF BLUBBER

Blubber regarded as a Heat Transmitter

Blubber is generally thought of as a protection against cold, but the integument of a homothermous animal is much more than a mere passive insulator. It actively controls the outward passage of heat, and so preserves the deep body temperature despite changes both in the rate of production of heat by the animal, and in the thermal characteristics of the environment.

In whales, no less than in terrestrial animals, is such a control of heat loss necessary. Variation in the rate of swimming must be accompanied by a considerable variation in heat production; and whereas most whales probably undertake irregular wanderings which carry them over at least a moderate range of temperature, the rorquals undergo a twice-yearly migration between polar and tropical waters, involving a temperature change of no less than 25°C .

The difference in the environment of whales and terrestrial animals carries with it necessary differences in the methods of heat-regulation. The latter lose heat by three channels: evaporation, controlled by the sweat glands and breathing; and convection and radiation, controlled by the surface temperature which is in turn influenced by vaso-regulation. In addition, habitat selection plays an important role. A whale, on the other hand, cannot sweat or seek shelter, neither can it radiate to an environment which is within a degree or two of its own surface temperature. It can lose heat solely by 'forced' convection to the water flowing past it, and we have shown that blubber contains a blood-circulation by control of which this heat loss could be regulated. The system will now be considered in more detail.

Minimum Heat Transmission. It is known that in man exposed to cold the blood-flow through the skin is reduced almost to zero by constriction of the arterioles (Lewis, 1927; Forster *et al.*, 1946). It is not unreasonable to assume that this is also true of whales where similar arterioles exist, though it may be added that if a slight flow is always necessary for purposes of nutrition, the heat loss from this might be reduced by a return stream of blood through the 'accompanying venules'. This would short-circuit some of the heat and prevent it from being lost at the surface.

When the blood-flow through the blubber is negligible, heat loss is entirely due to passive conduction through the tissues and its rate is given by the expression:

$$\text{Rate of heat transmission} = \frac{\text{Blubber conductivity}}{\text{Blubber thickness}} \times \left(\frac{\text{Body temperature} - \text{Sea temperature}}{\text{Sea temperature}} \right).$$

The conductivity and internal temperature were found as follows:

(i) *Conductivity.* A sample of whale blubber (*Balaenoptera physalus*) about 1 ft. square was hard-frozen a few hours after the capture of the whale, and maintained in this condition for about five months during transport to, and storage in, this country. It was then kept at just over 0°C . for a week to thaw out and at air temperature (October) for $3\frac{1}{2}$ hours during transport to the National Physical Laboratory, where it was immediately put in the heat-conduction apparatus. The Laboratory report was as follows:

The sample of whale blubber submitted for the determination of the thermal conductivity was supplied in the form of two slabs each measuring 12 inches by 12 inches by $1\frac{1}{4}$ inches approximately. For the purposes of the test, the slabs were placed one on each side of a hot plate surrounded by a guard ring, the whole being clamped between two cold plates maintained at a constant temperature. The

temperatures of the hot and cold surfaces were measured by means of thermocouples. The heat input to the hot plate was obtained by observations of the watts dissipated in its heating coil. The results given in the table below refer only to the particular sample tested.

Whale Blubber

(Approximate density 65 lb. per cub. ft.)

Cold face temperature: 0°C .

Hot „ „ : 35°C .

Thermal conductivity : 0.00050 gr.-cal. per sq. cm. per sec. for
1 cm. thickness and 1°C . diff. in
temp.

(ii) *Internal Temperature*. That of a Blue Whale (*Balaenoptera musculus*) was measured 60 minutes after death, the thermometer being placed in a deep cut in the epiaxial muscle, level with the flipper, immediately after the blubber had been removed from that part. Three readings all gave the same result: 35.5°C ., which is likely to be nearer the true value than Laurie's figure (1933) of 35.1°C ., based on the measurement of twenty whales at a land station.

Substituting these values for the conductivity and internal temperature in the above expression, we have:

$$\text{Heat transmission (cal./sq. cm./sec.)} = \frac{5 \times 10^{-4}}{d} (36 - t_e),$$

where d = thickness of blubber (cm.) and t_e = environmental temperature ($^{\circ}\text{C}$.).

Two examples may now be considered. For a porpoise in which $d = 1.8$ cm., living in a sea at $t_e = 10^{\circ}\text{C}$. (e.g. around the British coast):

$$\begin{aligned} H &= \frac{5 \times 10^{-4}}{1.8} (36 - 10) \\ &= 72 \times 10^{-4} \text{ gm.-cal./sq. cm./sec.} \\ &= 260 \text{ kilo-cal./sq. m./hr.} \end{aligned}$$

For a rorqual in which $d = 8$ cm., living in a sea at $t_e = 0^{\circ}\text{C}$., the heat loss is 81 kilo-cal./sq. m./hr. The significance of these figures will appear when it is recalled that the basal metabolism of a wide range of terrestrial animals (including horse, man, pig, dog, and hen) is almost constant and equal to 45 kilo-cal./sq. m./hr.—a figure which has come to be regarded as generally applicable though its physiological basis is unknown (see Krogh, 1941). If this figure also applies to the whale then we must conclude that neither the porpoise nor the rorqual, in the environments considered above, can afford to remain at rest: despite their blubber they would suffer a net loss of heat. It also follows that for the heat loss to be reduced to 45 kilo-cal./sq. m./hr. in any whale at rest (independent of size or species), a layer of blubber with an average thickness of 14 cm. would be required, a thickness which is

actually encountered in the largest rorquals, and in the right whales (*Balaenidae*), whose 'rightness' as a target for the harpoons of an earlier age was in part due to their sluggishness compared with the rorquals. We are, of course, aware that some authorities are unwilling to grant to the above figure for basal metabolism the dignity of a physiological constant. Benedict (1938) puts forward a relation making metabolism per unit area increase with length, quoting figures as low as 12 kilo-cals./sq. m./sec. for the dwarf mouse, and as high as 85 kilo-cal./sq. m./sec. for the elephant. His conclusion may be criticized on the ground that it is based on very few data from animals at the two extremes of size; but even if it is correct it quite fails to endow a small whale such as a porpoise with a basal metabolism nearly large enough to compensate for its heat loss in temperate seas.

Maximum Heat Transmission. This will be achieved when the blubber arterioles are fully dilated and the mass flow of blood to the surface is maximal. Then it is most likely that much more heat is lost from the blood than through the blubber, so the latter can be ignored in comparison. The interesting consequence is that maximum and minimum heat losses are virtually independent: the achievement of a low minimum by the development of a thick layer of blubber does not affect the maximum which will depend on the maximum mass flow of blood through the superficial vessels and their spacial arrangement. This arrangement may be contrasted with that found among the majority of terrestrial homotherms where the insulation (fur or feathers) overlies the vascular tissue, so that an increase in the thickness of the former must reduce the maximum as well as the minimum heat loss.

BLUBBER REGARDED AS AN ENERGY RESERVE

It is well known that the blubber of the southern rorquals shows a seasonal variation in thickness, being thinnest at the beginning of the southern summer when the whales are returning to high latitudes after wintering nearer the equator. This may be regarded as effecting a necessary increase in the minimum rate of heat loss corresponding to the warmer environment. But it has been suggested that the whales find little to eat in the tropics and subsist on the stored energy of their blubber. A simple calculation shows that this suggestion is quite plausible. According to Mackintosh and Wheeler (1929, p. 370) the reduction in thickness is 0.1 per cent. of the body length. Using the previously found expression $A = 0.39/l^2$ for the surface area, and assuming the density of blubber is unity and its energy content 9 kilo-cal./gm., it follows that the energy liberated during 6 months is at the rate of ($l^3 10^{-6}$) watts. Now basal metabolism at 45 kilo-cal./m.²/hr. demands $22^2 \times 10^{-3}$ watts; so it follows that a 0.1 per cent. reduction in blubber thickness would just pay for the basal metabolism of a 20-m. whale, while it would afford larger animals extra energy for movement.

This conclusion is not intended to be taken quantitatively; but it does give qualitative support to the idea that the blubber of rorquals functions as a significant energy reserve.

While this work was being done I was successively Whaling Inspector and Discovery Biologist in the Factory Ship *Empire Venture* (1945-6), and the recipient of a Senior Research Grant from the Department of Scientific and Industrial Research (1946-7). I am most grateful for the facilities which these appointments placed at my disposal. I also wish to acknowledge the willing assistance of the Director and Staff of the Scottish Marine Biological Association in securing and injecting the porpoise; and the readiness of the National Physical Laboratory to accept for conductivity measurements a specimen possessing somewhat unwelcome characteristics.

SUMMARY

1. The gross morphology, micro-anatomy, and histology of the blubber of the porpoise (*Phocaena phocaena*) and the rorquals (*Balaenoptera* spp.) are described.

2. If the surface area is given by Kl^2 , l being the overall length, then K is 0.39 in *Phocaena* and 0.35 in *Balaenoptera*, excluding fins and flukes.

3. Blubber consists of the whale's epidermis, dermis, and hypodermis. The hypodermis is relatively very thick and almost exclusively fatty, and in the species studied merges into the dermis which is mostly composed of white fibres. It extends into the epidermis as 'dermal ridges', from which the papillae arise. In the epidermis can be recognized: *stratum germinativum*, divided into the deep cylindrical cells and the more superficial prickly cells; and *stratum corneum*.

4. The vascular system is composed of arterioles running up to the base of the epidermis, giving rise to twigs which run up the dermal ridges to supply the capillaries in the papillae; and venules collecting twigs from the ridges and running down through dermis and hypodermis, connecting in the dermis with a venous plexus. Small 'accompanying venules' run with the arterioles to the base of the epidermis.

5. The conductivity of blubber is 0.00050 gm.-cal./sq. cm./°C./cm. and the deep body temperature is about 36° C. Thus in temperate and polar waters most whales lose heat at a greater rate than the basal metabolic rate of land homotherms, even when the blood-flow through the blubber is negligible. It is suggested that whales need to keep swimming in order to keep warm.

6. The vascular system in the blubber provides a mechanism for regulating heat loss.

7. It is shown that the energy liberated by the reduction in blubber thickness suffered by rorquals in the southern hemisphere during the winter is sufficient to meet at least a significant part of their total needs.

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EXPLANATION OF PLATE I

(All the preparations are of *Phocaena phocaena*)

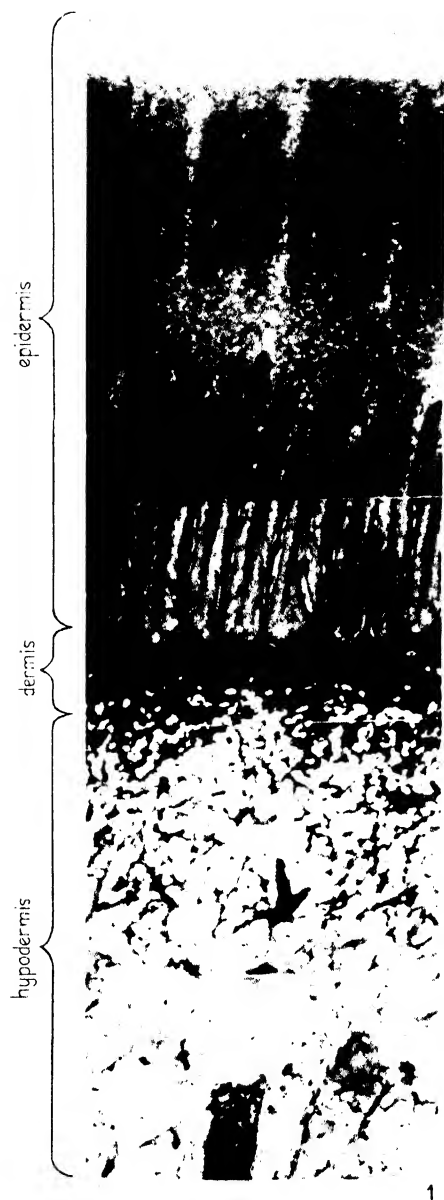
FIG. 1. Section through the epidermis, dermis, and superficial part of the hypodermis. Fixed in 5 per cent. formalin, several days *post mortem*. 100μ frozen sections, stained with 1 per cent. aniline blue, W.S. ($\times 29$)

FIG. 2. Horizontal section through epidermis, showing dermal papillae with capillaries dark with blood. Fixed in 5 per cent. formalin, several days *post mortem*. 100μ frozen sections, unstained. ($\times 52$)

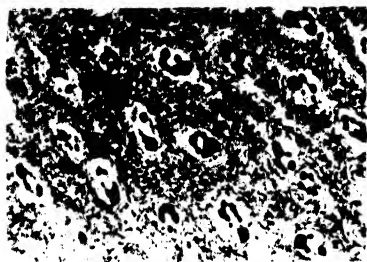
FIG. 3. Horizontal section through epidermis, showing dermal ridges with venules dark with blood. Treatment as (2). ($\times 52$)

FIG. 4. Horizontal section through base of epidermis and dermal ridges, showing large venule. Fixed as (2); 200μ frozen section, unstained. ($\times 52$)

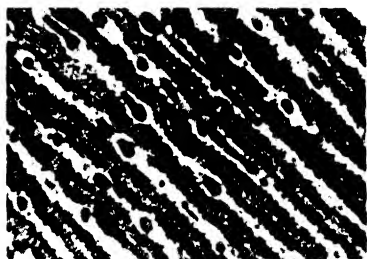
FIG. 5. Horizontal section through dermis, showing venous plexus. Fixed as (2); 200μ frozen section, unstained. ($\times 52$)



1



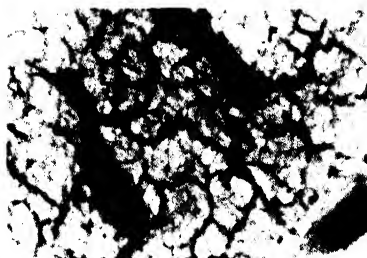
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4



5

D. A. PARRY—PLATE I

Heliospora n.g. and *Rotundula* n.g., Gregarines of *Gammarus pulex*

BY

HELEN PIXELI GOODRICH

(From the Department of Zoology and Comparative Anatomy, Oxford)

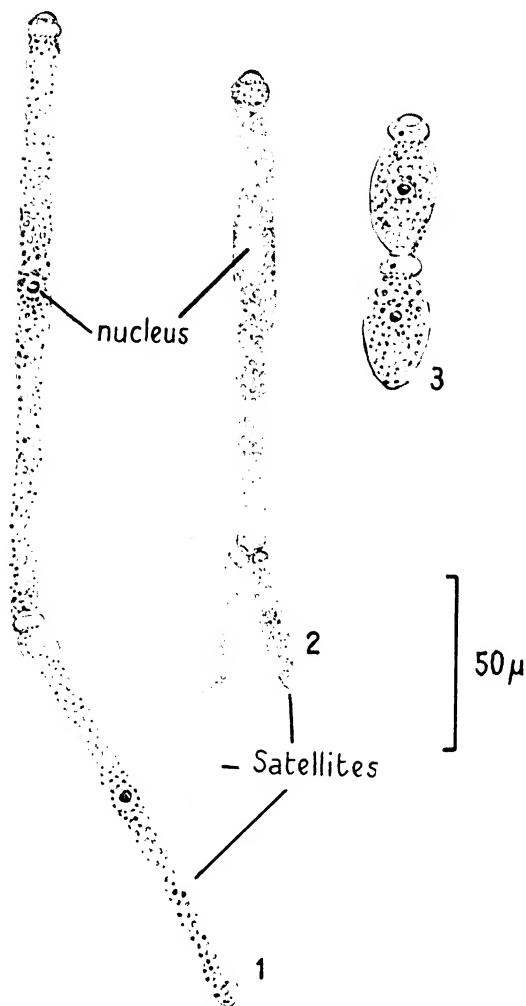
With one Plate

THE gregarines of *Gammarus pulex* L. have long been known, in fact they were some of the earliest of these Sporozoa to be observed (Text-figs. 1-3). However, so far, no adequate description has been given of the gametes and spores which are the most important stages from the systematic point of view.

Lady Muriel Percy, who worked in this Department from 1925 to 1929, helped in the investigation of spores by collecting infected *Gammarus* from different localities and isolating the Gregarine cysts for observations. From her notes and figures together with mine, which extend at intervals over the last quarter of a century, I have endeavoured to elucidate the life-histories of these two gregarines for which, I regret to say, it has been necessary to establish new genera as above.

Apparently Siebold was the first to study the trophozoites—some time in the early eighteen-forties. He did not describe them in his paper of 1839, as has been erroneously stated by several writers, but gave Kölliker permission to incorporate his description in a postscript to his (Kölliker's) paper of 1848. There, one can find quite recognizable figures (29 α and β) of a thread-like form, *G. longissima* and (γ) of a shorter, more rotund form. Siebold and others (Labbé, 1899) were uncertain whether the latter was a young stage of the former.

They were separated in the same year, 1848, by Frantzius, who attached the long thread-like gregarine of *Gammarus pulex* to the genus *Didymophyes* (just created by Stein for *D. gigantea* of *Oryctes* and *D. paradoxa* of *Geotrupes*), leaving the short form occurring with it as *G. gammari*?. He was followed by Diesing, 1859, who definitely stated that he excluded the latter from *G. longissima* and consequently this species *G. gammari* is now ascribed to Diesing owing to the uncertainty of Frantzius and the original observer Siebold. There is no doubt that '*longissima*' and '*gammari*' are their correct specific names. To find correct generic names has been more difficult. The thread-like form, even without knowledge of its cyst, could not be kept in the genus *Gregarina*, and Poisson (1921*b*), realizing that it also had no place in the genus *Didymophyes*, changed it to one *Uradiaphora* suggested by Mercier (1912*b* and *c*; see also p. 32 below) for a gregarine found in the fresh-water shrimp



TEXT-FIG. 1. *Heliospora longissima* Sieb. A pair in syzygy.

TEXT-FIG. 2. A living primitive of the same with 2 satellites attached.

TEXT-FIG. 3. *Rotundula gammari* Diesing. A pair of associates.

Figs. 1 and 3 fixed Bouin's mixture and stained iron haematoxylin.

Atyaephyra at Nancy. Various characteristics of this genus, some contradictory, were given in Mercier's four papers of 1911 and 1912, e.g. 1912c, p. 198: 'La syzygie à maturité sexuelle mesure de 5-700 μ de longueur. Kystes ovoïdes de 38-44 μ de long.' The latter and the spores appear more like those of *Rotundula* (see below, p. 33). The most surprising characteristic given by Mercier and said to be distinctive and from which he derived the

name, was the presence of a constriction towards the end of the deutomerite of the satellite forming 'une petite queue'. This the author suggested might be to prevent another trophozoite 'de venir troubler l'harmonie du couple'. This tail was said to be shaped like an appendix and to degenerate. Nothing of this sort has ever been seen by us in either of the gregarines of *G. pulex*. Sometimes the satellite itself of the long form is abnormally small and such a small one as well as one of more normal size are sometimes both attached to the primite (Text-fig. 2). We have evidence to show that both of these satellites may sometimes form gametes which undergo syngamy with those from the primite. Enough has, I think, been said, together with the characteristics to be given below, to show the necessity of making a new genus for this long-known thread-like gregarine of *G. pulex*, especially when the spores, considered the most diagnostic feature of gregarines, were found to be quite different from those of any known form.

These spores are provided with equatorial ray-like processes of the episporal coat and the name *Heliospora* therefore seems to be suitable.

Characteristics of *Heliospora* n.g.: Elongated, septate (polycystid) Gregarines having more or less spherical spores each provided with equatorial ray-like processes of the episporal coat.

Type species *Heliospora longissima* (Siebold) (see K  lliker, 1848) from gut of *G. pulex* L. Specific characteristics:

1. Elongated, filiform trophozoites, precociously associated.
2. No intracellular stage.
3. Small, button-shaped epimerite.
4. Epimerites and septa retained until the sporonts roll up for encystment.
5. Cyst approximately spherical, wall thin, transparent, and easily ruptured.
6. Gametes anisogamous, ♂ with delicate flagellum.
7. Spores slightly flattened at poles and at the equator the episore is produced into 6 long ray-like processes.

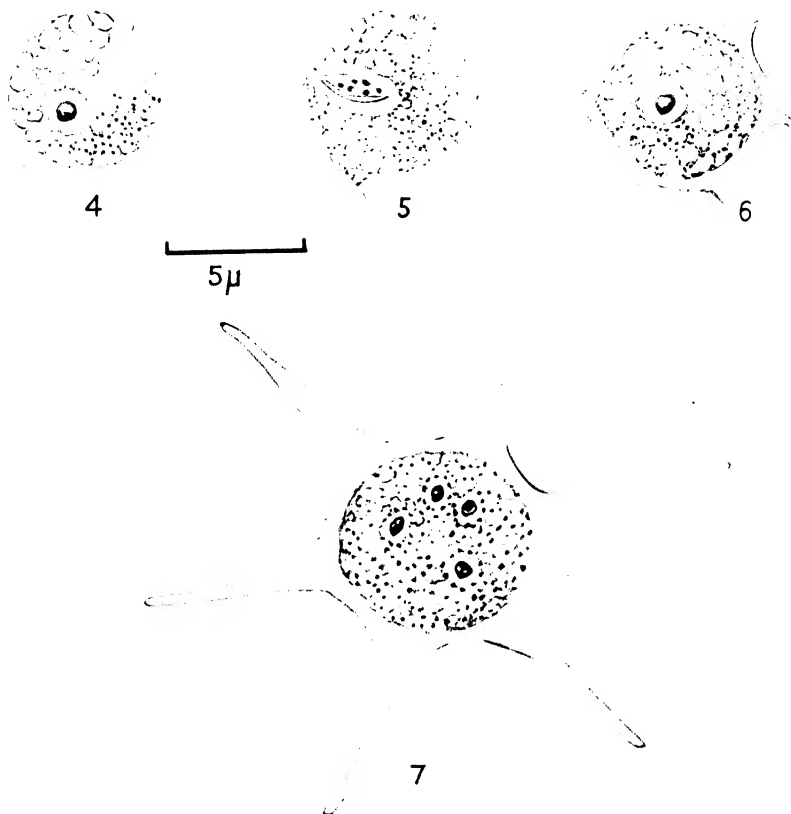
The thread-like trophozoites of *Heliospora longissima* may be up to 228μ long, but they naturally vary in length according to their state of contraction. They are sometimes only 8μ in diameter but may be double this width, especially in the region of the nucleus. The primite is often the longer but by no means always and our attempts to distinguish sex by intra-vitam stains have not given conclusive results. However, by watching development carefully, the male gametes have appeared to come from the smaller associates.

No intracellular stage has been found but young forms from 7.5 to 40μ or so long may be free in the lumen or cling to epithelial cells just as a satellite often presses its epimerite into the deutomerite of the primite to form a pair of different sex precociously. Many of these syzygies may be clustered together as so characteristic of gregarines. Towards the posterior end of the mid-gut, long pairs may be found folding themselves up with rather jerky movements,

finally forming cysts. These are $55-85\mu$ in diameter and through the thin wall the folds of the cuticle may be visible for some time. These cysts, often attached to a peritrophic membrane, pass out shortly before the host moults, or they may be left in the proctodaeum and cast off in the moult. With the cysts, trophozoites at various stages may be eliminated also but in our experience they do not develop into cysts outside. The loss of many parasites in this way at moulting seems to account for the poor infections often found, and the habit shrimps have of eating their moults may also destroy some unripe cysts. Perhaps the best way of finding cysts is to place a single pair of *Gammarus* in a bowl of water shortly before young are due to be expelled from the ♀'s brood pouch; after this the ♂ helps her to moult and, *if infected*, cysts may be found adhering to the bowl. To catch the ♂ moulting is more difficult. When the cysts are evacuated in water, they sink and attach themselves to the bottom of the bowl by an adhesive secretion. By means of a needle or fine brush they can be lifted and mounted in a hanging drop, but on reaching the surface they, of course, tend to be detached and should be caught in a small pipette before again reaching the bottom of the bowl. With care these thin-walled cysts can be mounted unharmed and watched developing under high magnification through their transparent walls. In this way the accompanying time-table was worked out, see diagram (pl. I). There does not seem to be any definite time of day or night for moulting nor for the extrusion of cysts, but the one drawn (stage 1) was one of the first to be studied and we have been able to foretell the time of the dance on other occasions. It begins some 11 hours after the elimination of the cysts. It will be seen that the partition between the two associates (stages 1 and 2) soon disappeared but the cytoplasm again collected round each nucleus until division started some 3-4 hours later. During the next 3 hours these nuclei arranged themselves on the surface and there continued their division. About 3 hours later again some cytoplasm collected round each nucleus and the gametes became separated, and soon after the slight movement began. So far as we could make out only the smaller gametes (♂) had active movement, and this was due to a very fine flagellum which persisted in the zygote (Text-figs. 4 and 5), but was difficult to fix. However, when left to themselves the dance ended in an hour or rather less and development then proceeded. After a further 12-24 hours the cyst would burst and liberate ripe spores—the average time for this appeared to be 18 hours—so that the approximate time for the development of ripe spores would be 30 hours from the extrusion of the cyst from the host. The spores ($7-8\mu$ in diameter) are slightly flattened at the poles. At first the equatorial ray-like processes (nearly 10μ long) wrap round the lower poles of the spores (Text-fig. 8) but as they float away these processes gradually extend themselves, appearing from above like the rays of the sun (Text-fig. 9).

The episporal processes are shown up very clearly by staining with Steven's blue-black ink, as has often been found before (Pixell Goodrich, 1929). They start to develop (Text-fig. 6) while the synkaryon is still resting and are apparently full length at the 4-nuclear stage (Text-fig. 7).

The 8 sporozoites (Text-fig. 10), each about 6μ long, seem to escape through an operculum, but we have not succeeded in observing this process either in the fore gut or outside. There is a residuum of large refringent granules.



TEXT-FIG. 4. Male gamete with nucleus and flagellum.

TEXT-FIG. 5. Zygote with synkaryon and flagellum.

TEXT-FIG. 6. Sporocyst from above with resting nucleus and developing episporal rays.

TEXT-FIG. 7. The same with 4 nuclei and 6 rays.

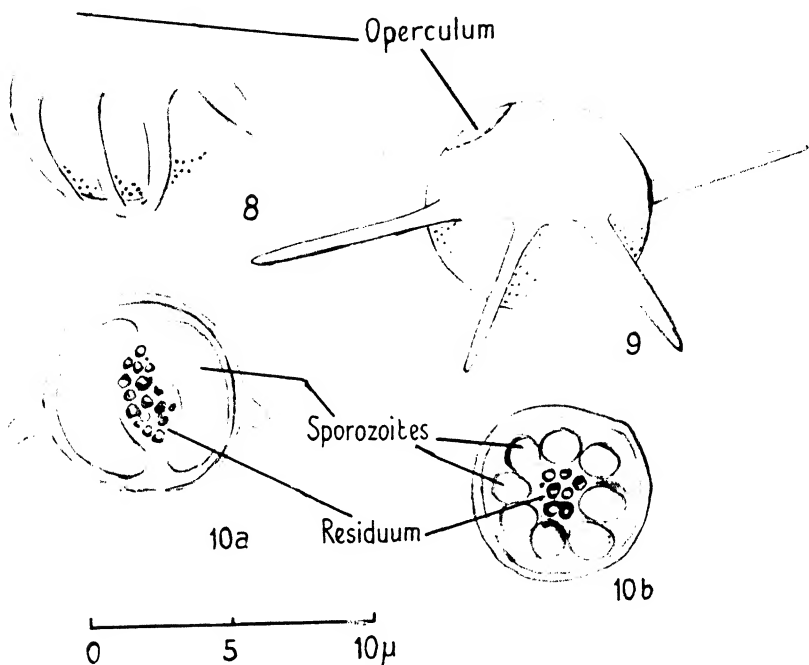
Figs. 4-7 fixed Schaudinn's solution and stained iron haematoxylin.

Rotundula gammari (Diesing, 1859)

This short somewhat rotund polycystid gregarine (Text-fig. 3) has been even more difficult to fit into any genus so far described and to prevent confusion the name *Rotundula* has been given. Superficially the trophozoite, of course, does show some resemblance to such members of the genus *Gregarina* as *G. ovata*, &c. However, it differs fundamentally in having no

large cyst with sporoducts through which the characteristic adhering spores emerge in long chains.

The generic difficulty arose chiefly from the fact that in 1908 Mavrodiadi gave a preliminary note in a small Russian paper of a polycystid gregarine



TEXT-FIG. 8. Spore just emerged from cyst with 5 of the episporal rays still folded underneath it.

TEXT-FIG. 9. Spore with expanded rays—drawn from one side.

TEXT-FIG. 10. Optical sections of spores (a) longitudinal, (b) transverse—showing sporozoites and residual protoplasm.

Figs. 8–10. Spores of *Heliospora longissima* drawn from the living.

in *Balanus* to which he gave the name *Cephaloidophora*. His figures (13) of an elongated spore and (7–11) of sporozoites and trophozoites growing deeply embedded in the tall epithelial gut cells of the *Balanus* are quite clear. In spite of this Mercier (1911) called a gregarine of the Caridine from Nancy *Cephalodophora cuenoti*. It had no intracellular stage and the next year (1912b) he removed it from Mavrodiadi's genus, saying (p. xliii, footnote): 'Je ne connais pas le mémoire original de Mavrodiadi écrit en russe.' He then called it *Uradiophora*, a genus proposed by him (1912b and c) to which I have referred above (pp. 27–8) as being an ill-defined genus to which Poisson (1921b) attached the long thread-like gregarine of *G. pulex*.

Unfortunately, in the intervening years, other gregarines were crowded into the genus *Cephaloidophora* (see Watson Kamm, 1922), many of them apparently having no better claims than Mercier's to be there, and one cannot help suspecting that some of the authors, like Mercier, had not studied Mavrodiadi's Russian paper.

In 1911 Léger and Duboscq described a polycystid gregarine from *Gammarus marinus* at Roscoff which is very like this *G. pulex* parasite *R. gammari*. Strangely enough this well-described parasite was also placed in Mavrodiadi's genus under the name of *Cephaloidophora maculata* after being removed from *Frenzelina* which was found to be preoccupied.

I should like to propose that it be changed to *Rotundula maculata* (L. and D.), and it would then be the type species of this genus, only differing apparently in having a marine host and possibly some of its young stages intracellular.

Rotundula n.g. Generic characteristics: Septate (polycystid) gregarines tending to rotundity, mostly free in the lumen of the gut but they may be temporarily attached to or between the epithelial cells.

Epimerite button-like and persistent.

Protomerite rounded with a nucleus in young stages.

Precocious association.

Cyst oval or round: wall covered with a gelatinous layer: no sporoducts.

Spores spherical or subspherical, small.

Specific characteristics of *R. gammari* (Diesing, 1859) from gut of *G. pulex*.

Everything about this gregarine is more or less round—a button-like epimerite, which, however, flattens out before encystment, the protomerite is almost spherical, vacuolated in the living and has 1 or sometimes 2 or 3 chromatic granules (nuclei) which, however, generally disappear before encystment.

The deutomerite may become almost spherical before encystment. Its nucleus is spherical and contains 1 large spherical karyosome. Cysts 40–50 μ , not adhesive. Spores small, more or less spherical with a faint equatorial suture; 8 sporozoites and a refringent residuum.

Very small specimens, from 6 μ long, have been seen free in the lumen or mixed with the cellular debris cast off with the peritrophic membranes from mid-gut and there is no doubt that they associate very early. I have seen a specimen lying between the shallow epithelial cells as shown in Léger and Duboscq's fig. V (1911). One would expect that the epimerites might cling to cells in this way as they do to the deutomerites of other specimens to form syzygies. In fact this does not appear to be a real intracellular stage as these distinguished protozoologists seem to assume.

A pair of trophozoites have often been seen folding over one another and encysting towards the end of the mid-gut. When eliminated the cysts are more difficult to find in the water than those of *H. longissima*.

They are, for one thing, smaller—seldom more than 50 μ —and they have an outer gelatinous wall which swells in water so that the cysts become about

the same density as the water and probably remain suspended: also they are not adhesive. The development when we have been able to follow it out appears to be just like that described by Léger and Duboscq (1911, fig. VI) for *C. maculata*. The gametes are roundish, the male, barely 4μ in diameter, having a fine flagellum. The spores are more or less spherical, only 5 or 6μ in diameter: they have a fine equatorial suture along which no doubt splitting takes place in the ripe spore and liberates the 8 small sporozoites, but we have not seen these escape.

Rotundula can be distinguished from *Cephaloidophora* in having a rounded not elongated spore and by most of its stages being free in the lumen of the gut.

Poisson (1921a) named a gregarine he had found in *Echinogammarus berilloni*, *Cephaloidophora echinogammari*, and proceeding to the observation of the gregarines of *G. pulex* (1921b) thought he discovered the same species there and made the curious suggestion that this was only present in *G. pulex* when this shrimp was living in close association with *Echinogammarus*.

This cannot possibly be true, for in this Oxford region, where we have studied *Gammarus* for some 25 years, there is no *Echinogammarus* nor other species of Gammarid than *G. pulex* (we are indebted to Charles Elton for confirming this). It may be infected with either or both the gregarines described here, viz. *H. longissima* (Siebold) or *R. gammari* (Diesing). No satisfactory record of any other than these two gregarines has been found throughout the 100 years that they have been studied. Therefore, if *Echinogammarus* contains the same parasite, it must be the species named *G. gammari* by Diesing in 1859.

In *Orchestia bottae*, recently found (Cain and Cushing, 1948) in numbers on the banks of the Thames here, there is an even more rotund gregarine which resembles *Rotundula* in its vegetative stages, but we have not studied its development. Poisson and Remy (1925) called it *C. orchestia*. It seems likely that this, as well as *C. echinogammari* Poisson, will be found to fit into the genus *Rotundula* as well as Léger and Duboscq's *C. maculata*.

In fact, the gregarines of most Amphipods will, I suspect, be found closely related to these long-known parasites of *G. pulex*.

I thoroughly agree with Poisson (1924, p. 247) that most of the gregarines of Crustacea require revision and their gametes and spores to be carefully studied: the cysts, as he remarks, are often difficult to collect. However, it cannot be too strongly emphasized that the important stages contained in them must be known before their proper systematic position can be determined.

In conclusion, I should like to express my thanks to Professor A. C. Hardy, F.R.S., who has put every facility at my disposal for continuing my work in his Department.

SUMMARY

From this study of the unknown stages in the life-histories of the two polycystid gregarines, which have been known for just 100 years in *Gam-*

marus pulex L., it is concluded that the long thread-like one should be *Heliospora longissima* (Siebold) and the short rounded one *Rotundula gammari* (Diesing), and that some other Amphipods have the same or similar gregarines.

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TIME

TIME

STAGES

NOON



1



5

7 p.m.

12-30



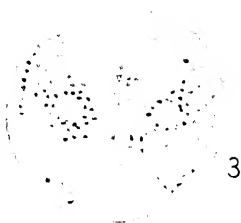
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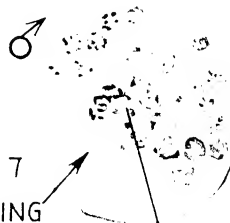
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10-30

1-45



3



11 p.m.

 BEGINNING
DANCE
ENDING

RESIDUUM

4 p.m.



4



12

MIDNIGHT

8

 50 μ

CYSTS AT VARIOUS STAGES

1. Cyst at extrusion (noon, on this occasion).
- 4-6. Cysts with nuclear division proceeding.
7. Cyst with ♂ and ♀ gametes—only a few of these are shown—sometimes the cyst is so full that only slight movement can be detected before syngamy.
8. Cyst showing a few zygotes moving round the residual protoplasm.

Studies on Abnormal Mitosis induced in Chick Tissue Cultures by Mustard Gas ($\beta\beta'$ -Dichlorodiethyl Sulphide)

BY

A. F. W. HUGHES

(*Sir Halley Stewart Fellow*)

AND

HONOR B. FELL

(*Foulerton Research Fellow, Royal Society*)

(*From the Strangeways Research Laboratory, Cambridge*)

With three Plates

INTRODUCTION

THAT mustard gas causes structural changes in chromosomes was first shown indirectly by the important genetical experiments of Auerbach and Robson (1944, 1946, 1947), who found that by exposing adult male *Drosophila* to mustard-gas vapour a wide range of mutations was produced. Direct cytological evidence was provided by Koller (1947), who demonstrated various types of chromosomal abnormality in the pollen grains of *Tradescantia* after treatment with different concentrations of the vapour. These observations have recently been elaborated and extended by Darlington and Koller (1947).

In work done during the war for the Chemical Defence Research Department of the Ministry of Supply, Fell and Allsopp (1948) noticed profound disturbances of the mitotic process in the cells of tissue cultures growing in a medium containing 5–100 γ /c.c. of mustard gas. Later they found similar abnormalities in the regenerating epidermis of mice treated with repeated applications of minute quantities of the agent. Mitotic abnormalities have also been described by Gillette and Bodenstein (1946) and Bodenstein (1947) in amphibian embryos treated with a nitrogen mustard compound.

Recent optical developments have made it possible to study cell division in living material in much greater detail than hitherto. Hughes and Swann (1948) investigated the anaphase movement in cultures of normal chick osteoblasts, using both phase-contrast and polarized-light microscopy, and are accumulating more information about the mitotic spindle by these methods. It seemed desirable to extend this work with similar observations on different types of abnormal mitosis induced by chemicals and other agents, in the hope that the results might shed further light on the physiology of normal cell division.

The extensive literature on the pathology of mitosis has been reviewed by Politzer (1934). Abnormal mitoses in tissue cultures have been produced by

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very varied experimental means. Among the most notable contributions to this subject are the beautiful direct observations of reversible changes in living mitotic cells *in vitro* made by M. R. Lewis (1923, 1933*a* and *b*, 1934). The effect on dividing cells in culture of many different chemicals has been studied: various acids (M. R. Lewis, 1923; Bauer, 1923), reduced neutral red (M. R. Lewis, 1923), carbon dioxide (Mottram, 1928), ether (Kemp and Juul, 1930; Rosenfeld, 1932), potassium iodide (Razzesi, 1932), ammonia (Rosenfeld, 1933), auramine, urethane, methyl sulphonal, sodium cacodylate, colchicine and some of its derivatives, quinine, atropine, aconitine (Ludford, 1936), and of certain carcinogens and related hydrocarbons (Hearne Creech, 1939). The action of heat (Kemp and Juul, 1930; M. R. Lewis, 1933*a*), of hypotonic culture medium (M. R. Lewis, 1934), and of radiation (Strangeways and Oakley, 1923; Strangeways, 1924*a* and *b*; Lasnitzki, 1943; and others) have also been investigated. Abnormal mitosis may be produced merely by the addition of stale plasma to the culture medium (Strangeways, 1924). Cinema films of pluripolar mitosis *in vitro* have been made by W. H. Lewis (1932) and by G. Gey (1947).

The observations described in the present paper refer to cells growing in a medium containing low concentrations of mustard gas (pure $\beta\beta'$ -dichlor-diethyl sulphide). The results were obtained partly by the analysis of cinema films of the living cells made by phase-contrast microscopy, and partly from the cytological study of fixed and stained cultures; these two methods of approach were found to be complementary, and each demonstrated features of abnormal mitosis not shown by the other.

A. H. was responsible for the cinematography and the quantitative data obtained from the films and H. B. F. for the tissue culture and the observations on fixed material. The general analysis of the films and their interpretation were the joint work of both authors.

MATERIAL AND METHODS

Tissue Culture. Fragments of the frontal bones from 11- to 12-day fowl embryos were cultivated in a mixture of equal parts of fowl blood-plasma and tissue extract made with Tyrode from 11- to 12-day chick embryos. The explants were grown in hanging drop preparations on $1\frac{1}{4}$ in. square No. 2 coverslips over $3 \times 1\frac{1}{2}$ in. hollow-ground slides.

The cultures were incubated for 2-3 days, by which time the original bone fragment had become surrounded by a halo of migrating cells consisting mainly of osteoblasts. The tissue was then transferred to medium containing mustard gas in one of three concentrations: 12.5, 25.0, and 50.0 γ /c.c. The agent was introduced into the culture medium in the following way. A solution of mustard gas in absolute alcohol was prepared and a small quantity was added to a known volume of plasma in such a way that the plasma contained double the amount of the agent that was required for the final culture medium. A drop of this plasma solution was then placed on a No. 1 coverslip and mixed with an equal drop of embryo extract. The explant was transferred

to the mixture before it clotted, and for cinematography the coverslip was mounted on a special type of culture vessel; the application of phase-contrast illumination to tissue cultures and the culture chamber devised for this purpose have been described elsewhere (Hughes and Swann, 1948). Cultures required for fixation and staining were mounted on hollow-ground slides in the usual way. All the preparations were incubated for 24–48 hours before use.

In one experiment the explants were transferred to normal medium, incubated for 24 hours, then opened, and a drop of serum containing 50 γ /c.c. of mustard gas was deposited on the tissue. After this treatment the cultures were mounted on the special chambers mentioned above and examined either immediately or after 24 hours' further incubation.

Cinematography. The phase-contrast objective ($\times 95$) and condenser used for this work were supplied by Messrs. Cooke, Troughton & Simms, Ltd. (see Hughes and Swann, 1948). For the photomicrography of living cells in tissue cultures this apparatus should be used in conjunction with sensitized film of maximum contrast. This is particularly important for cells treated with mustard gas in which the contrast of the chromosomes is much below normal, probably owing to a diminished content of nucleic acid.

After many different types of film had been tried with the generous collaboration of the research staff of Messrs. Kodak, Ltd., a special 16-mm. negative of extremely high contrast was chosen, known as 'film for ciné-photomicrography'. The contrast in the photograph is greater than in the direct image, so that the cellular detail can be seen much better in a print than by direct observation through the microscope. The use of this film in the study of living cells will be described elsewhere.

The ciné records were studied exhaustively by projection in both directions, by examination frame by frame, and by comparing paper enlargements of selected series of frames. The sequences described below were analysed by means of all three methods. The rates of anaphase movement were obtained by measuring the distances between the daughter chromosome groups in successive frames projected on paper (Hughes and Swann, 1948).

Fixation and Staining. In most of the experiments some of the cultures were used for ordinary cytological study. They were fixed for 4–5 min. in Maximow's solution (10 parts Zenker's fluid: 1 part formol: 1 part 2 per cent. osmium tetroxide) freshly prepared for each occasion, washed overnight in distilled water, then treated with alcoholic iodine and washed with 70 per cent. alcohol. Some of the cultures were stained by Feulgen's method. Others were hydrolysed for 8 min. at 60° C. as for the Feulgen technique and then stained for 10–15 min. in well-ripened Ehrlich's haematoxylin; this method gave a very clear picture of the cells and of the chromosome structure and was particularly suitable for photography; the distribution of the stain was precisely the same as with the Feulgen technique. Preparations stained with haematoxylin without previous hydrolysis were much inferior in clarity to the hydrolysed specimens. The stained cultures were dehydrated, cleared, and mounted whole in Canada balsam.

For certain purposes fixation for 3–4 min. in Zenker's fluid without acetic acid followed by hydrolysis and staining with Ehrlich's haematoxylin was useful. This method was particularly suitable for demonstrating the smaller micronuclei in the many multi-nucleate cells which mustard gas produced in the cultures (Pl. III, fig. 31); it also rendered the spindle of mitotic cells very distinct (Pl. III, fig. 30).

RESULTS

Normal Mitosis as seen by Phase-contrast Microscopy

In normal living osteoblast cultures the intermitotic cell (Pl. I, fig. 3) is much flattened and usually of triangular or spindle-shaped outline; the oval nucleus, which is rather paler than the cytoplasm, contains one or more irregular nucleoli which appear nearly black. In the cytoplasm are seen the dark mitochondrial filaments, many small granules, and the highly refractile fat globules. Both the cell and its contents are in continual slow movement.

When prophase begins the cytoplasmic processes are largely withdrawn, the nucleoli vanish, and faintly grey, diffuse chromosomes materialize throughout the nuclear area. At the same time the nuclear membrane disappears, but cytoplasmic inclusions remain outside the nuclear area until late anaphase. The chromosomes contract, become increasingly distinct, and assume a radial orientation in the plane of the coverslip (Pl. I, fig. 1a); they probably lie in this plane as the mechanical result of the flatness of the cell. Whether the spindle has already begun to form is not yet known, and since it is therefore uncertain whether this stage should be regarded as late prophase or early metaphase, we have termed it the radial stage.

As the cell becomes more nearly spherical, the chromosomes rotate from the plane of the coverslip to one at right angles to it, presumably under the influence of the spindle elements, but in normal cells the spindle is indistinct with phase-contrast microscopy and the details of its formation cannot be seen. During metaphase (Pl. I, fig. 1b) the chromosomes move to and fro in the equatorial region of the spindle with unsynchronized linear motion (W. H. Lewis, 1939; Hughes and Swann, 1948). Without warning the chromatids suddenly separate and pass quickly to opposite poles (Pl. I, fig. 1c, d). Details of the anaphase movement have been described by Hughes and Swan (1948).

About 3 min. after the beginning of anaphase, the granules and mitochondria of the surrounding cytoplasm bulge into the inter-zonal region and constriction into daughter cells begins. 'Bubbling' of the peripheral cytoplasm, spreading from the poles to the equator (cf. Chambers, 1938), may occur at any stage of mitosis but always becomes increasingly vigorous during telophase (Pl. I, fig. 1e). The contrast of the chromosomes falls during telophase so that usually nothing is clearly visible in the nuclear area until 12–20 min. after anaphase, by which time the nuclear membrane and nucleoli are present and the daughter cells are flattening (Pl. I, fig. 1f). A connecting thread of cytoplasm persists for a very variable time and then snaps.

The stages of normal mitosis as they appear in fixed and stained cultures are shown in Pl. III, figs. 12-17.

The Effect of Mustard Gas on Mitosis

I. General Effects on the Cells (Table I)

In living cells grown in the presence of small quantities of mustard gas and examined by phase-contrast microscopy, the cytoplasmic structures and particularly the mitochondria are abnormally distinct, but, as stated above, the contrast of the chromosomes is subnormal. Owing to the high contrast of the negative used, however, the general form of the chromosomes can be distinguished in cinema films of the living cells, but suitably fixed and stained preparations are required for a more precise study of chromosome structure.

At the higher concentrations of mustard gas the volume of the cell, as judged by its surface area, is increased. The fat content, even after 48 hours' cultivation, is abnormally low at all three concentrations, and the cytoplasm may be free of all but the smallest lipid granules. These small lipid granules are seen to be in Brownian movement by direct observation, whereas in the normal cell movement can only be directly appreciated in a minority of the globules. From this it may be inferred that the water-content of the cells treated with mustard gas is abnormally high and the viscosity of the cytoplasm relatively low.

The degree of mitotic disturbance caused by the presence of mustard gas in the culture medium varies in the same culture from a slight deviation from the normal to great irregularity. The proportion of extreme abnormalities is naturally much larger at the two higher concentrations, but even at the 12.5 γ /c.c. level a few greatly distorted mitotic figures are seen (Pl. III, fig. 28).

Three main types of abnormal mitosis may be distinguished, which are bipolar, tripolar, and apolar, respectively; they will be considered in detail in the next section.

II. Observations on the Three Main Types of Abnormal Mitosis

1. *Bipolar Mitosis.* At the lowest concentration of mustard gas (12.5 γ /c.c.), some mitoses are nearly normal. This is illustrated by one of the ciné records:

Record 1 (Pl. I, fig. 2). In this film mitosis, and in particular the radial stage, is unduly prolonged (see Table II), but otherwise division proceeds normally.

The abnormal bipolar mitoses may be divided arbitrarily into two groups, in one of which (group A) the abnormality is much less extreme than in the other (group B); in life the dividing cells of group B are usually larger than those of group A.

Mitoses of group A are characterized by the delayed arrival of certain chromosomes at the equatorial plate or their failure to reach it (Pl. III, figs. 18 and 19), by lag at anaphase and telophase (Pl. III, figs. 20-22), and by the formation of one or more micronuclei derived from the lagging chromosomes in addition to two daughter nuclei of nearly normal size (Pl. III, figs. 23 and 24).

TABLE 1. Summary of Observations, mainly from Cinema Records, of Mitotic Abnormalities produced by Mustard Gas

N. = normal R.I. = record incomplete

		Bipolar			Tripolar	Apolar
		A	B			
Number of examples recorded by cinema	Nearly normal	12:5 v/c.c.: 3 25:0 v/c.c.: 3 50:0 v/c.c.: 2 50:0 v/c.c.: 1 (in serum)	25:0 v/c.c.: 3 50:0 v/c.c.: 1 50:0 v/c.c.: 2 (in serum)		25:0 v/c.c.: 2	25:0 v/c.c.: 1 50:0 v/c.c.: 3
Illustrations	..	Pl. I, figs. 2, 4, 5 Pl. III, figs. 18-24	Pl. I, fig. 6 Pl. II, fig. 8 Pl. III, figs. 25-29		Pl. II, figs. 9, 10 Pl. III, fig. 30	Pl. I, fig. 7 Pl. II, fig. 11 Pl. III, figs. 31, 32
Duration	4 cells: N. 2 cells: R.I.	Fig. no. 2, 5 2 cells: prolonged 1 cell: R.I.	No record	Fig. no.	Fig. no. 10 1 cell: prolonged 1 cell: R.I.	Fig. no. 11 1 cell: prolonged 3 cells: R.I.
Radial movement	4 cells: N. 2 cells: R.I.	Fig. no. 2, 5 1 cell: ++ 1 cell: N. 1 cell: R.I.			Fig. no. 10 1 cell: ++ 1 cell: R.I.	Fig. no. 11 1 cell: ++ 3 cells: R.I.
Duration	3 cells: prolonged 1 cell: N. 2 cells: R.I.	Prolonged up to twice N.	4 cells: prolonged 2 cells: R.I.	6, 8	1 cell: R.I. 1 cell: no distinct meta-phase	9 1 cell: R.I.
Delay or failure in reaching metaphase plate	..	1 cell: +	Obscure in records, ++ in fixed cells	6, 8	1 cell: ++	No distinct metaphase
Linear movements on spindle	N.	+	1 cell: ++ 3 cells: ++ 1 cell: N. 1 cell: R.I.	6, 8	1 cell: +	
Condition of chromosomes	N.	N. or slightly beaded	Incomplete contraction, pronounced beading		May be abnormally short and thick	Granules or distorted rods and filaments
Movement of anaphase groups	N. rate	N. rate	N. rate		1 cell: N. rate	No recognizable anaphase
Lag	..	2 cells: + 1 cell: -	4, 5 5 cells: ++ 1 cell: -	6, 8	++	
Type of cleavage	2 daughters	2 daughters	2 daughters		1 cell: 3 daughters 1 cell: at first 3 daughters, but 2 by secondary fusion	No cleavage
Rate of cleavage	N.	About half N. rate	About half N. rate		About half N. rate	
Cytoplasmic streaming	N.	2 cells: 2 of N. size + micronuclei 1 cell: 2 of N. size	4 cells: ++ 1 cell: N. Several nuclei of various sizes	6, 8	++ Several nuclei of various sizes	+++ Many nuclei of different sizes
Daughter nuclei	2 of N. size					

Some of the chromosomes appear normal in stained preparations, but in others the nucleic acid charge is localized in granules, the intergranular material being nearly colourless when stained by Feulgen's method or by Ehrlich's haematoxylin (Pl. III, fig. 20). Some interesting features of these mitotic abnormalities are seen in two of the ciné records (Records 2 and 3).

TABLE 2. *Showing Phase Times in Minutes of Bi- and Tri-polar Mitoses*

N.N. = nearly normal

Treatment	Type of division	Pro-phase	Meta-phase	Beginning of anaphase to			Record no. in text	Remarks
				Early cleavage	End of cleavage	Nucleoli in daughter cells		
8 controls	Normal	..	c. 4	2.3-5.0	3.5-6.0	11-20	..	
12.5 γ /c.c.	N.N.	> 2.4	9.4	3.6	7.0	13.2	..	
"	N.N.	3.0	6.8	13.4	..	
"	3.8	6.6	10.0	..	
25.0 γ /c.c.	N.N.	..	> 6.7	3.4	6.2	9.1	..	
"	N.N.	> 4.7	9.9	3.7	6.1	9.4	..	
50.0 γ /c.c. (serum)	N.N.	> 4.2	21.4	3.8	9.4	11.4	..	Nucleoli still present when record begins
12.5 γ /c.c.	A	> 36.6	6.4	4.8	9.6	18.2	1	
"	A	..	> 7.2	5.8	8.4	13.0	2	
"	A	> 12.6	10.6	1.8	6.8	12.4	3	
25.0 γ /c.c.	B	12.0	..	3.9	5.9	7.8	..	
"	B	..	16.2	4.1	9.5	10.8	4	
"	B	..	13.0	3.2	5.5	13.9	..	
50.0 γ /c.c. (serum)	B	..	10.8	3.8	7.0	12.0	..	
"	B	..	> 135.0	3.3	6.2	17.2	5	
25.0 γ /c.c. (serum)	Tri-polar	..	> 1.8	5.0	9.5	10.7	6	

Record 2 (Pl. I, fig. 4). This cell shows a simple lag of chromosomes at anaphase within the spindle area; it finally divides into two daughter cells, one of which contains a single nucleus, while the other forms one nucleus of nearly normal size and two micronuclei.

Record 3 (Pl. I, fig. 5). The cell is in prophase when the film begins. Most of the chromosomes pass to the equator in the normal way, but two fail to join the others and remain near one pole of the spindle (Pl. I, fig. 5*b*). Within 2 min., however, the tardy chromosomes are drawn to the equator (Pl. I, fig. 5*c*) and soon afterwards anaphase begins. Several chromosomes on each side of the metaphase plate do not divide and separate with the rest, but lag behind at the equator (Pl. I, fig. 5*d*). As the peripheral cytoplasm bulges into the interzonal region preparatory to cleavage, the lagging chromosomes from each side are pushed towards each other and are finally brought together by the equatorial constriction (Pl. I, fig. 5*e*); this lateral form of lag will be discussed in more detail below. After anaphase the fate of the laggards is obscured by the bubbling of the cytoplasm. Eventually one daughter cell is seen to contain a single large nucleus and the other one large nucleus and a micronucleus.

The divisions of group B are much more abundant at the higher concentrations than at the 12.5 γ /c.c. level and the mitotic abnormality is an exaggeration of that described above in group A. The spindle seems relatively normal, but in stained preparations the chromosomes appear very deficient in nucleic acid and have not contracted properly; there is very pronounced lag at anaphase and the daughter cells contain more micronuclei than are formed in the divisions of group A.

In prophase (examined in fixed preparations only) the filaments are somewhat vaguely defined and stain lightly as compared with the normal; they often vary in thickness along their length and are sometimes beaded; they may be unevenly distributed in the nuclear area and in places entwined to form long, tangled, granular skeins (Pl. III, cf. figs. 12 and 25). At metaphase (Pl. III, figs. 26 and 27) the chromosomes are seen to vary enormously in size and appearance, some being minute granules, others long, beaded, and often attenuated filaments; in Feulgen preparations, the beads on the long chromosomes are Feulgen-positive while the rest of the thread is nearly colourless, indicating severe nucleic acid deficiency. Other chromosomes have an irregular outline and the Feulgen-positive material is aggregated in lumps here and there on the nearly unstained thread. Some chromosomes may fail to reach the equatorial plate and lie in the cytoplasm.

In many of the cells at metaphase most of the chromosomal material is aggregated into one or more large tangled skeins (Pl. III, figs. 26 and 27) similar to, but usually larger and more compact than, those sometimes seen at prophase (Pl. III, fig. 25). The filamentous structure of these masses varies in distinctness in different cells; in some they are quite clearly composed of entwined, beaded threads, while in others they appear as almost homogeneous bodies. These masses may lie on each side of the spindle at the equator or freely in the cytoplasm (Pl. III, fig. 27). Sometimes the large chromatinic masses move to the surface of the cell, where a strictly localized bubbling of the cytoplasm takes place (Pl. I, fig. 6a-c; Pl. III, fig. 27).

Anaphase (Pl. III, fig. 28) is characterized by a pronounced equatorial lag, usually of several chromosomes, though the two main daughter groups pass to the poles at the normal rate (Text-fig. 1). This failure of individual chromosomes to move normally to the poles of the spindle is of two types: a simple median lag, usually of small chromosomes, within the spindle, and a lateral lag, to which reference has already been made, which is associated with a somewhat diffuse structure of the metaphase plate, especially when the chromosomes are incompletely contracted. In the latter types, in which the equatorial plate is disproportionately large for the spindle, the long lateral chromosomes and sometimes large chromosomal masses (Pl. III, fig. 28) fail to move during anaphase and remain flanking the interzonal region, the interior of which is clear except for the small chromosomes undergoing median lag (Pl. II, fig. 8b-e).

When cleavage begins, constriction of the interzonal region pushes the mitochondria and the lateral lagging chromosomes into an axial position, mid-

way between the groups of daughter chromosomes; here they remain, until the completion of cleavage incorporates them in one or other of the daughter cells where they form micronuclei (Pl. II, fig. 8e-g). Such lagging filamentous chromosomes often produce a bridge of chromatin uniting the two daughter cells, and expanding at either end into an oblong or pear-shaped nucleus. Sometimes this bridge is surprisingly long and attenuated (Pl. III, fig. 29) indicating considerable plasticity of the chromosomes.

The multinucleate condition of the daughter cells in group B is not always due entirely to the lagging chromosomes. In some cells the daughter chromosomes of the two anaphase groups are very loosely arranged (Pl. III, fig. 28) and instead of forming a single large nucleus, give rise to a nest of micronuclei; there is some evidence both from the films and from fixed material that there may be secondary fusion in such nests, so that one or more larger nuclei are later formed. It is probable that chromosomal material which fails to reach the spindle at metaphase (Pl. III, fig. 27) also forms micronuclei at telophase.

Most of the phenomena described above are illustrated by two of the film records (Nos. 4 and 5).

Record 4 (Pl. II, fig. 8). The cell is in metaphase when photography begins. During anaphase a group of chromosomes moves to each pole in the usual way, but several chromosomes, in some of which a beaded structure is very distinct, lag behind (Pl. II, fig. 8c). When the cell constricts at cleavage, chromosomes previously lateral to the spindle and possibly not incorporated in the metaphase plate, together with some of the lagging chromosomes mentioned above, are pushed into an axial position (Pl. II, fig. 8d, e). Thus at telophase there are three groups of chromosomes in course of nuclear reconstruction—one equatorial and two polar (Pl. II, fig. 8f). Each polar group forms a single oval nucleus of normal size and appearance which moves freely in the cytoplasm. The equatorial group, however, produces a bouquet of small pear-shaped nuclei in one daughter cell (Pl. II, fig. 8g), which are unable to move freely until liberated from their attachment to the interzonal strand by the completion of cleavage. Finally, one daughter cell is seen to be mononucleate, while the other contains one nucleus of normal size and several micronuclei derived from the interzonal group.

Record 5 (Pl. I, fig. 6). In this cell both the spindle and the chromosomes are very distinct. When the record begins, there are several large, irregular granular masses and small chromatinic granules and filaments distributed over the spindle (Pl. I, fig. 6a). The smaller structures display very active, unsynchronized linear movement up and down the spindle, but the larger masses drift outwards, apparently under the influence of cytoplasmic currents. The filaments alternately stretch and retract as they move up and down, and the larger chromosomal bodies continually change shape. Some of the larger masses, though lying in the cytoplasm, retain their connexion with the spindle, but the largest body moves right out of the spindle area and slowly works its way to the surface of the cell, where an active and strictly localized bubbling

begins (Pl. I, fig. 6a-c). A small cytoplasmic protuberance is formed at this point into which the chromosomal body passes (Pl. I, fig. 6c). Metaphase was enormously prolonged and anaphase did not take place until 135 min. after the beginning of observation.

The rate of anaphase movement is normal, but the groups of daughter chromosomes represent a relatively small proportion of the total chromatin content of the cell (Pl. I, fig. 6e). Much of the material is incorporated in the large irregular bodies described above; other chromosomal structures remain scattered near the poles of the spindle, never having reached the equatorial plate, while others again lag behind during anaphase. When cleavage begins both daughter cells show a spiral streaming of the cytoplasm which is much more active in one cell than in the other. Eventually this mitosis produces two daughter cells, each containing many medium-sized and small nuclei (Pl. I, fig. 6f, g).

2. *Tripolar Mitosis.* In tripolar mitosis there may be a typical triradiate metaphase (Pl. II, fig. 9a and Pl. III, fig. 30), or a recognizable metaphase may be entirely omitted, the cell passing straight from the radial stage to a tripolar anaphase and telophase (Pl. II, fig. 10). An example of both types of tripolar division was recorded by the cinema (Records 6 and 7).

Record 6 (Pl. II, fig. 9). A regular triradiate metaphase with active linear movement up and down the spindle axes is seen. Not all the chromosomes are incorporated in the spindle area, many being scattered throughout the surrounding cytoplasm. Suddenly the cell enters anaphase (Pl. II, 9b) and a group of chromosomes passes to each of the three poles, leaving a fourth group of laggards moving irregularly in the centre of the cell (Pl. II, fig. 9c). Meanwhile the outline of the cell becomes triangular and the cytoplasm begins to bubble. Bubbling increases in violence and the cell divides into three multinucleate daughters (Pl. II, fig. 9d-e).

Record 7 (Pl. II, fig. 10). This shows a much less regular tripolar division. An incomplete ring of chromosomes (Pl. II, fig. 10a) in active radial movement passes directly into a tripolar anaphase (Pl. II, fig. 10b), without forming a recognizable metaphase. All the chromosomal material seems to be in the form of granules; some are double and the two constituents are pulled apart at anaphase. Telophase, with violent bubbling and streaming of the cytoplasm, produces three daughter cells united by narrow bridges (Pl. II, fig. 10c), but before cleavage is complete two of the daughter cells reunite, so that finally one large and one smaller cell are formed, both of which are multinucleate (Pl. II, fig. 10d).

3. *Apolar Mitosis.* In mitosis of the apolar type there is no recognizable metaphase or anaphase. In some cells the chromosomal material is in the form of distorted filaments and rods, but in others most of it appears as granules (Pl. III, fig. 32), some of which are fairly large but others so small as to be only just visible. These dust-like particles are often arranged in radiating lines, but it is impossible to see whether they are discrete bodies or minute beads on a continuous unstained thread. Usually these cells also

contain one or more much larger chromatinic bodies, in some of which a closely tangled filamentous structure is distinguishable in fixed preparations while others appear as homogeneous globules (Pl. III, fig. 32). This modified radial stage may continue for a fairly long time, to be succeeded by a phase of nuclear reconstruction in which the scattered chromosomal material forms a host of nuclei of widely varying sizes (Pl. III, fig. 31). There is no cleavage of the cytoplasm.

Cinema films were made of three cells of this type, two of which are described below (Records 8 and 9):

Record 8 (Pl. II, fig. 11). When the film begins, radially arranged granules of chromatin are seen in active radial movement. Suddenly this scattered group of chromosomal bodies is churned round by a rapid spiral streaming of the cytoplasm which continues for a short time and then gradually subsides. The cell begins to flatten and a number of nuclei develop (Pl. II, fig. 11d). Finally, a single large multinucleate cell is formed (Pl. II, fig. 11e); one nucleus is much larger than the rest and appears to be the fusion product of several smaller nuclei.

Record 9 (Pl. I, fig. 7). In this cell the chromosomal material is very dispersed (Pl. I, fig. 7a), but two large lumps of chromatin are clearly seen at one stage (Pl. I, fig. 7b). At first the cell is fairly quiescent, then an active spiral movement of the cytoplasm, associated with violent hubbbling, begins (Pl. I, fig. 7b); this commotion gradually dies away and a single cell results in which about 17 nuclei were counted in life (Pl. I, fig. 7c).

III. Time Relations in Abnormal Mitosis (Table 2).

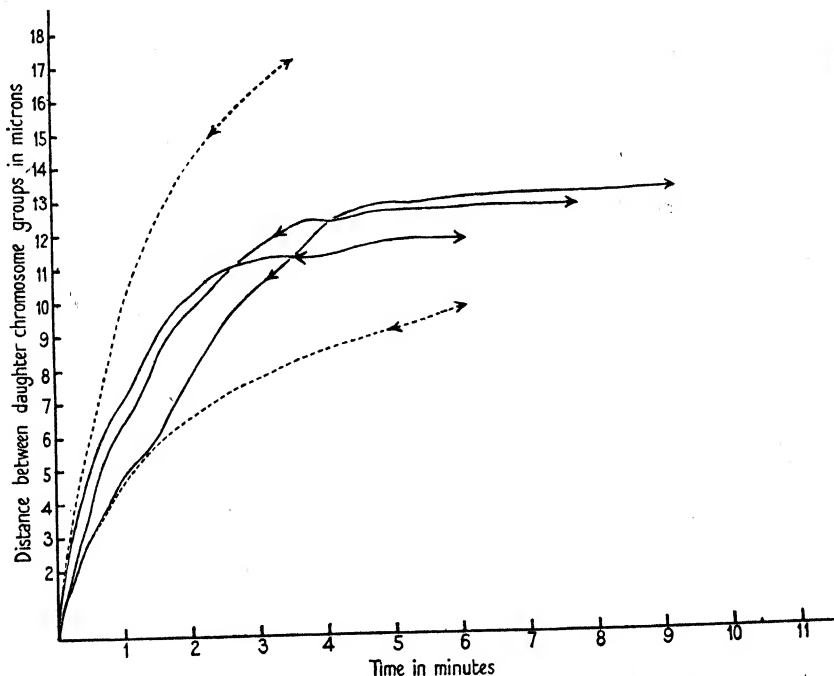
The duration of each mitotic phase in cells dividing under the influence of mustard gas was measured from the photographic records. The figures obtained, with comparable data from normal material, are presented in Table 2.

The most sharply defined stages in mitosis are the beginning of anaphase and the end of cleavage; the transition from the radial stage to metaphase is more gradual but can usually be estimated to within half a minute. Photography began either at prophase or metaphase, and the duration of the phase in which the cell was first observed is indicated as more than ($>$) the recorded time. The late phases of chromosome division are reckoned from the beginning of anaphase; the beginning of cleavage is defined as the constriction inside the cell of the interzonal region of the spindle or, when this is not visible, as the first appearance of an external cleavage furrow.

In general, mitosis is prolonged by the influence of mustard gas, and prophase (Pl. I, fig. 2) and metaphase (Pl. I, fig. 6) in particular may be greatly protracted. The maximum duration of a prophase that was known to be succeeded by a metaphase was >36.6 min. Twice we followed what appeared to be a prophase for 78 min. ($12.5 \gamma/\text{c.c.}$ mustard gas) and 144 min. ($50 \gamma/\text{c.c.}$) respectively, but during these periods the cells did not enter metaphase and observation was discontinued.

The maximum duration of a metaphase that we have recorded was >135 min. (Pl. I, fig. 6), a period several times as long as that of any other metaphase that we have studied. Such an extreme prolongation seems to be exceptional.

Anaphase proceeds at the normal rate. Curves of anaphase movement plotted against time were obtained from our cinema records (Text-fig. 1);



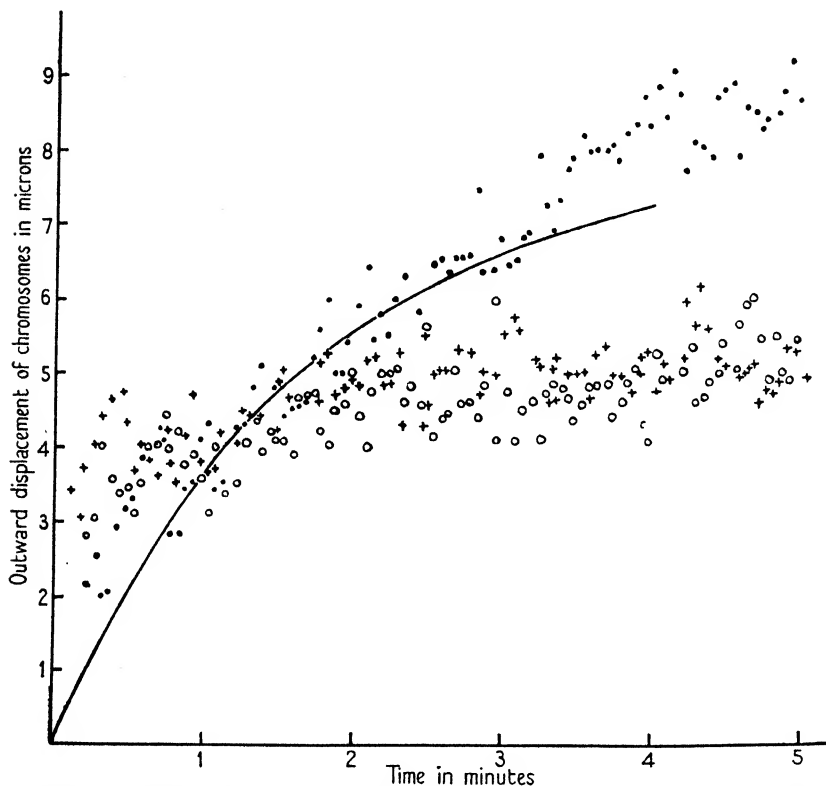
TEXT-FIG. 1. Anaphase curves of three bipolar mitoses from cultures treated with 50 γ /c.c. mustard gas, compared with curves (dotted) which represent the two extremes of a group of eight mitoses in normal osteoblasts. The beginning and end of cleavage is indicated.

when compared with curves from similar records of normal osteoblast cultures (Hughes and Swann, 1948) they are seen to fall within the normal range as regards rate of movement, distance traversed by the chromosomes, and general shape. At the higher dosage levels, however, the curves tend to move towards the lower limit of the normal range. The curves in the text-figure do not, of course, refer to the lagging chromosomes, which either remain stationary or move more slowly than the others.

Cleavage is somewhat, but not greatly, protracted, but the reconstruction of daughter nuclei, as indicated by the interval between the beginning of anaphase and the first appearance of nucleoli, is not greater than normal and sometimes may possibly even be less. Comparison with the corresponding period in the division of normal cells, however, is complicated by the fact that reconstruction of the daughter nuclei is less obscure in the

treated than in the untreated cells, so that possibly the formation of nucleoli is visible at an earlier stage in the abnormal mitoses.

A normal rate of chromosome movement at anaphase with a somewhat prolonged cleavage are also found in cells growing in normal medium, but subjected to the intense illumination necessary for polarized light microscopy



TEXT-FIG. 2. Measurements of the outward displacement of chromosomes in a tripolar mitosis in an osteoblast culture treated with 25 γ /c.c. mustard gas (Record 6). The three symbols refer to each of the three half-spindles. The continuous curve is the average displacement of one group of daughter chromosomes in eight normal osteoblast mitoses.

(Hughes and Swann, 1948), or to γ -radiation, as well as in cultures treated with other chemicals (Hughes, unpublished).

As stated above, we have one cinema record of a fairly orderly anaphase movement in a tripolar mitosis (Pl. II, fig. 9). In this cell a group of chromosomes remains in the centre, and the distance between this central group and each of the three daughter groups during anaphase was plotted (Text-fig. 2). It will be seen that the rate of movement of each chromosome group in the tripolar anaphase corresponds roughly with that of a single daughter group in a normal bipolar anaphase.

DISCUSSION

The effects of mustard gas on mitosis *in vitro* are not specific. Such minor abnormalities as failure of one or more chromosomes to reach the equatorial plate, slight lag at anaphase, and the production of one or two micronuclei, are produced by many mildly unfavourable conditions. The more severe abnormalities described above most nearly resemble those resulting from irradiation (Strangeways and Oakley, 1923; Strangeways, 1924*b*; Lasnitzki, 1943), though they are also rather similar to those caused by ammonia (Rosenfeld, 1933).

Some of the phenomena we have described are essentially the same as those observed by Koller (1947) and Darlington and Koller (1947) in the pollen grains of *Tradescantia* treated with mustard gas, though mitosis seems to have attained a more extreme abnormality in the tissue cultures than in *Tradescantia*. Darlington and Koller were chiefly concerned with the breakage of chromosomes in relation to genetical problems, while our interest has been focused on the effects of the agent on the general physiology of cell division. These authors stress the close resemblance between the changes produced in their material by mustard gas and by X-rays.

Darlington and Koller describe and figure nucleic acid deficiency ('nucleic acid starvation', Darlington and Koller, 1947), similar to that seen in many of the mitotic figures in the treated tissue cultures; they also record imperfect contraction of the chromosomes; fragmentation, sometimes into small particles ('minutes' and 'subminutes'); failure or delay in reaching the metaphase plate ('errors of congression'); lag in anaphase, and the formation of micronuclei from chromosome fragments. They noted a correlation between centromere defects and errors of chromosome movement in *Tradescantia*, and it is probable that in the osteoblast cultures failure or delay of certain chromosomes to take part in the normal movements of metaphase and anaphase are due to defects in the attachment of the chromosomes to the spindle elements.

Whether chromosome breakage and reunion such as that seen in *Tradescantia* occurs also in our cultures is not known; we hope to extend our investigations to amphibian tissue cultures which are much more favourable for detailed cytological study than those of the chick.

Bodenstein (1947), in his interesting studies of the effects of a nitrogen mustard compound on amphibian development, describes cytological abnormalities in the ectoderm of *Amblystoma* embryos, which resemble those seen in the osteoblast cultures treated with mustard gas. He records the enlargement of those cells which normally divide actively, 'metaphases with irregularly arranged chromosomes' and anaphase lag; in the ectoderm, as in the tissue cultures, normal and abnormal mitotic figures occurred in close proximity. Multinucleate cells appeared in the ectoderm 7-8 days after exposure to the agent when mitosis had ceased; Bodenstein believes that the nuclei of these cells were formed by the fragmentation of enlarged interphase nuclei. In our material such cells were seen to arise by abnormal mitosis. There is

also strong cytological evidence that in mouse skin treated with repeated applications of mustard gas in very dilute solution the many multinucleate cells which appeared in the regenerating epidermis were the result of abnormal mitosis (Fell and Allsopp, 1948). While it is of course possible that the ectodermal cells of the *Amblystoma* embryos reacted rather differently from those of mouse epidermis or of chick osteoblast cultures, it would be interesting to know whether anything similar to the apolar mitosis described above (cf. Pl. I, fig. 7, Pl. II, fig. 11, Pl. III, fig. 32) was present in the amphibian tissue. The interpretation of such grossly abnormal mitotic figures is difficult in material which precludes the direct observation of the living cells.

Precisely how mustard gas disturbs the physiology of dividing cells is not clear. The agent is known to have several biochemical effects: on proteins in general (Banks *et al.*, 1946), on nucleo-proteins (Berenblum and Schoental, 1947), and on carbohydrate metabolism through the inactivation of hexokinase (Dixon and Needham, 1946). Which of these effects operate in living cells exposed to very low concentrations of the agent, and to what extent, remains to be discovered.

Study of the cinema records emphasizes the fact that the various phenomena of mitosis are not rigidly linked to each other, and that in the same cell one process may be grossly distorted, or even omitted, while another proceeds almost normally. Thus the chromosomes may be extremely abnormal in structure, as in group B of the bipolar divisions, and yet at anaphase a considerable proportion of them move to the poles of the spindle at the normal rate: in one of the tripolar mitoses described above there is no metaphase, but the cell is able to pass directly from the radial stage into a tripolar anaphase; in the apolar mitoses not only metaphase but anaphase and telophase also are omitted, but nevertheless the general cytoplasmic upheaval normally characteristic of these phases takes place and the scattered and distorted chromosomal material is able to form nuclei.

These observations suggest that in mitosis there are parallel series of reactions, in the chromosomes, in the spindle, and in the cytoplasm, which in normal division are closely co-ordinated but which under the influence of mustard gas and other agents may partially disengage and to some extent proceed independently of each other.

The authors are indebted to the Royal Society and to the Medical Research Council by whom the expenses of the investigation were defrayed. They also wish to express their thanks to Mr. V. C. Norfield for the photomicrographs on Pl. III and to their assistant Mr. L. J. King for his help with the tissue culture. They are also indebted to Dr. H. McCombie of the Department of Chemistry, Cambridge University, who kindly provided the mustard gas and to Dr. C. B. Allsopp for preparing the stock alcoholic solution.

SUMMARY

1. The cytological effects produced in cultures of embryonic fowl osteoblasts by low concentrations of mustard gas in the nutritive medium have

been studied both in the living cells by means of cinematography and phase-contrast microscopy, and in fixed and stained preparations.

2. Under the influence of mustard gas the movements of the cell and its contents are exaggerated and, especially at the higher concentrations, its water-content appears to be increased.

3. The treated cultures contain many abnormal mitotic figures which are more abundant and more distorted at the higher concentrations.

4. Three main types of abnormal mitosis have been observed:

(i) *Bipolar*. Group A: these cells are characterized by failure or delay of certain chromosomes in reaching the equatorial plate, by lag at anaphase and telophase, and by the formation from the lagging chromosomes of one or more micronuclei, in addition to two nuclei of normal size. Most of the chromosomes appear normal, but in some the nucleic acid charge is localized in granules. Group B: the mitotic abnormality is an exaggeration of that seen in group A. The spindle is relatively normal; the chromosomes fail to contract properly and have a beaded structure; some chromosomes may break up into small granules while others aggregate into large, granular, skein-like masses; there is an equatorial lag, usually of several chromosomes; multinucleate daughter cells are formed.

(ii) *Tripolar*. Two forms of tripolar mitosis have been observed: (a) a fairly regular triradiate metaphase plate was succeeded by a tripolar anaphase and the formation of three multinucleate daughter cells; (b) a recognizable metaphase was omitted, the cell passing straight from the radial stage (see p. 40) to a tri-polar anaphase and telophase.

(iii) *Apolar*. There is no recognizable metaphase, anaphase, or telophase; the chromosomal material has a radial orientation; in some cells it is in the form of distorted filaments and rods, while in others it appears as granules of different sizes. After a period of intense cytoplasmic turmoil the cell spreads out without cleavage and many small and medium-sized nuclei have been formed from the diffuse chromosomal material.

5. In the abnormal cells the duration of mitosis is prolonged; at the higher concentrations prophase and metaphase may last for 2 hours or more; when a spindle is formed, some of the chromosomes move apart during anaphase at the normal rate; cytoplasmic cleavage may occupy 2–3 times the normal period; reconstruction of the daughter nuclei proceeds at the normal rate.

6. The cytological effects of mustard gas resemble those of irradiation.

7. The observations indicate that many phenomena of mitosis, though normally closely co-ordinated, under abnormal conditions can to some extent disengage and proceed independently of each other.

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DESCRIPTION OF PLATES

The figures in Plates I and II are of living cells photographed by phase-contrast microscopy. All were enlarged to the same magnification ($\times 1000$) from single frames of ciné records. In each series the times are reckoned from that of the first picture. The photographs in Plate III are of fixed and stained preparations and were taken by Mr. V. C. Norfield, head assistant at the Strangeways Research Laboratory.

PLATE I

Fig. 1a-f. Stages in the normal division of an osteoblast; a. radial stage; b. 8½ min., end of metaphase; c. 9½ min., early anaphase; d. 12 min., late anaphase; e. 13½ min., telophase: general cytoplasmic bubbling; f. 22 min., reconstruction of daughter cells: nucleoli have appeared.

Fig. 2a-f. 12.5 γ /c.c. mustard gas in the culture medium. Bipolar mitosis. Prolonged prophase, division otherwise normal. a. radial stage; b. 32 min., early metaphase; c. 36½ min., end of metaphase; d. 40 min., anaphase; e. 45½ min., late telophase; f. 62½ min., apparently normal mononucleate daughter cells.

Fig. 3. Normal intermitotic cell. Note the two irregular nucleoli in the large oval nucleus, and the very refractile cytoplasmic fat globules.

Fig. 4a-e. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A) showing chromosome lag in anaphase. *a.* end of metaphase; *b.* 1½ min., early anaphase; *c.* 3½ min., late anaphase; *d.* 8 min., telophase; *e.* 32 min., daughter cells: in the left-hand cell one large nucleus and two micronuclei are forming.

Fig. 5a-i. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A) showing delay of two chromosomes in reaching metaphase plate and lag in anaphase. *a.* prophase; *b.* 6½ min., early metaphase: chromosomes \times not yet in equatorial plate; *c.* 17½ min., metaphase: \times nearly on the plate; *d.* 24 min., early anaphase: \times incorporated in right chromosome group, lateral lag of two small chromosomes (seen as small black rods at the equator); *e.* 27½ min., early telophase: lagging chromosomes being pushed into an axial position; *f.* 29½ min., late telophase; *g.* 35½ min., *h.* 44 min., *i.* 55 min., stages in nuclear reconstruction: in the left daughter cell a micronucleus develops as well as a normal nucleus.

Fig. 6a-g. 50 γ /c.c. mustard gas added in serum (see p. 39). Abnormal bipolar mitosis (Group B) showing large, granular chromosomal masses, one of which (\times) is expelled from the spindle, and the formation of two multinucleate daughter cells. *a.* metaphase: \times in spindle area; *b.* 5½ min., metaphase: \times leaving spindle area; *c.* 45 min., metaphase: \times in cytoplasmic bubble; *d.* 134 min., end of metaphase; *e.* 136½ min., anaphase; *f.* 139½ min., telophase; *g.* 153 min., multinucleate daughter cells: note wide range of nuclear size.

Fig. 7a-c. 25 γ /c.c. mustard gas. Apolar mitosis forming single multinucleate cell. *a.* diffusé chromosomal material: absence of spindle; *b.* 10 min., two chromosomal masses visible, violent cytoplasmic bubbling; *c.* 35 min., multinucleate cell.

PLATE II

Fig. 8a-g. 25 γ /c.c. mustard gas; abnormal bipolar mitosis (Group B) showing median and lateral anaphase lag, beading of the chromosomes and the formation of one mono- and one pluri-nucleate daughter cell. *a.* end of metaphase; *b.* 1½ min., early anaphase; *c.* 2½ min., anaphase: lagging beaded chromosomes; *d.* 3½ min., late anaphase: lateral lagging chromosomes being pushed into an axial position; *e.* 7½ min., telophase: lateral chromosomes now axial; *f.* 15 min., daughter cells: abnormal persistence of cytoplasmic bridge, in lower daughter cell, bouquet of micronuclei formed from lagging chromosomes; *g.* 20 min., daughter cells: one mono- and one pluri-nucleate.

Fig. 9a-g. 25 γ /c.c. mustard gas. Tripolar mitosis forming three multinucleate daughter cells. *a.* end of triradial metaphase; *b.* 1½ min., early anaphase; *c.* 4 min., late anaphase: some chromosomes left in the middle of the spindle area; *d.* 6½ min., telophase; *e.* 10 min., *f.* 16 min., *g.* 18 min., stages in the formation of three multinucleate daughter cells.

Fig. 10a-d. 25 γ /c.c. mustard gas. Irregular tripolar mitosis with no recognizable metaphase, forming two daughter cells. *a.* radial stage: incomplete ring of chromosomes; *b.* 10 min., tripolar anaphase; *c.* 14½ min., tripolar telophase; *d.* 28 min., coalescence of two of the three daughter cells.

Fig. 11a-e. 50 γ /c.c. mustard gas. Apolar mitosis forming single multinucleate cell. *a.* radial stage showing ring of chromosomes; *b.* 36 min.; *c.* 40 min., expansion of cell processes; *d.* 100 min., a nest of nuclei have been formed, note the nucleoli; *e.* 115 min., cell fixed and stained, the nest of nuclei are clearly seen. ($\times 1300$.)

PLATE III

All the photographs, except Figs. 29 and 31, were taken at the same magnification as that of Fig. 12. The cells shown in Figs. 30 and 31 are from preparations fixed in Zenker's solution without acetic acid; the rest are from cultures fixed in Maximow's fluid. Figs. 13, 14, 19, and 26 were made from preparations stained by Feulgen's method and the remainder from preparations hydrolysed as for the Feulgen technique and then stained with Ehrlich's haematoxylin.

Fig. 12. Normal prophase. ($\times 1700$.)

Fig. 13. Late normal prophase.

Fig. 14. Normal radial stage.

Fig. 15. Normal metaphase.

Fig. 16. Normal anaphase.

Fig. 17. Normal late telophase.

Fig. 18. 25 γ /c.c. mustard gas. Abnormal bipolar metaphase (Group A), showing delay of one chromosome in reaching the equatorial plate; note the beaded end of this chromosome.

Fig. 19. 25 γ /c.c. mustard gas. Abnormal bipolar metaphase (Group A), showing chromosome right outside the spindle area.

Fig. 20. 12.5 γ /c.c. mustard gas. Abnormal bipolar anaphase (Group A), showing chromosome lag. Note the beaded structure of some of the chromosomes indicating nucleic acid deficiency.

Fig. 21. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A); early telophase with single lagging chromosome.

Fig. 22. 12.5 γ /c.c. mustard gas. Abnormal bipolar telophase (Group A) with several lagging chromosomes.

Fig. 23. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A); late telophase with three lagging chromosomes in course of reconstruction into micronuclei.

Fig. 24. 12.5 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group A); daughter cells, one containing a single large nucleus and two micronuclei and the other one large and one small nucleus.

Fig. 25. 25 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group B); prophase (cf. Figs. 12 and 13) showing granular chromosomes of irregular shape and distribution; at \times the chromosomal material forms a granular, skein-like mass.

Fig. 26. 25 γ /c.c. mustard gas. Abnormal bipolar metaphase (Group B); most of the chromosomal material is included in two large, granular masses on either side of the fairly normal spindle. The chromosomes in the spindle area are very deficient in nucleic acid and irregular in form.

Fig. 27. 50 γ /c.c. mustard gas. Abnormal bipolar metaphase (Group B) similar to that seen in Fig. 26. This cell contains three large chromosomal masses, two of which are lateral to the spindle, while the third lies freely in the cytoplasm near the surface of the cell. Note the localized cytoplasmic bubbling in the neighbourhood of all three masses.

Fig. 28. 12.5 γ /c.c. mustard gas. Abnormal bipolar anaphase (Group B) showing two diffuse groups of daughter chromosomes and at the equator several headed lagging chromosomes and large chromosomal masses.

Fig. 29. 25 γ /c.c. mustard gas. Abnormal bipolar mitosis (Group B) showing multinucleate daughter cells united by a long chromatinic bridge, each end of which has expanded into a nucleus. ($\times 1150$.)

Fig. 30. 50 γ /c.c. mustard gas. Tripolar metaphase.

Fig. 31. 50 γ /c.c. mustard gas. Multinucleate cell derived from an apolar mitosis. ($\times 880$.)

Fig. 32. 50 γ /c.c. mustard gas. Apolar mitosis showing radially arranged, granular chromosomes and homogeneous chromatinic body.

A Dye Phosphate for the Histo- and Cytochemical Demonstration of Alkaline Phosphatase, with some Observations on the Differential Behaviour of Nuclear and Extranuclear Enzymes

BY

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(From the Chester Beatty Research Institute, Royal Cancer Hospital, London, S.W. 3)

With one Plate

INTRODUCTION

THE method most generally employed for the cytochemical localization of alkaline phosphatase is that due to Takamatsu (1939) and Gomori (1939). It depends upon the hydrolysis by the enzyme of β -glycerophosphate in the presence of calcium nitrate. Calcium phosphate is thus precipitated at the site of enzymic activity, and may be rendered visible by various procedures. The method is unsuitable for tissues containing preformed calcium phosphate, unless they be previously decalcified: furthermore, it involves a number of stages, some of which necessitate a highly critical timing (Danielli, 1946). Lastly, the method as at present employed is not readily adaptable to quantitative histochemical estimations. Another technique, developed mainly by Menten, Junge, and Green (1944), depends upon enzymic hydrolysis of a phenol phosphate, in the presence of a diazonium hydroxide: immediately upon liberation, the free phenol couples with diazonium hydroxide and precipitates *in situ* as an insoluble dye. This procedure is independent of the phosphate residue, and preformed calcium phosphate does not interfere. Nevertheless, the method involves a number of disadvantages, viz.:

- (a) Solutions of diazonium hydroxides are unstable and must be freshly prepared.
- (b) The temperature of incubation must be kept very low, thus denying the enzyme its optimal conditions.
- (c) Diazonium hydroxides exert an inhibitory effect upon the enzyme.

It would clearly be advantageous if a technique were available in which a highly coloured substance is released from a phosphate ester by the action of phosphatase. The properties required of the substance to be released from the ester, ideally, are as follows:

1. It shall be highly coloured.
2. It shall be highly insoluble.
3. It shall not tend to crystallize in the tissue, but remain amorphous: at least, the crystals shall be submicroscopic.



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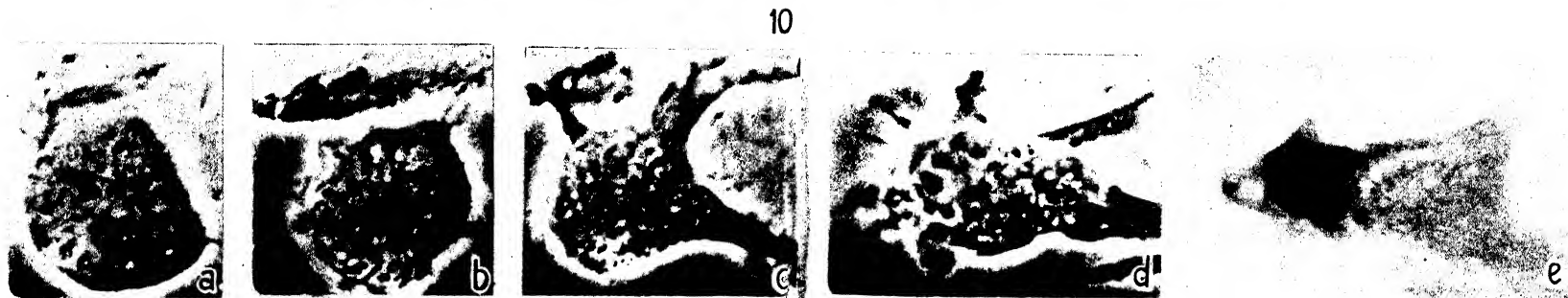
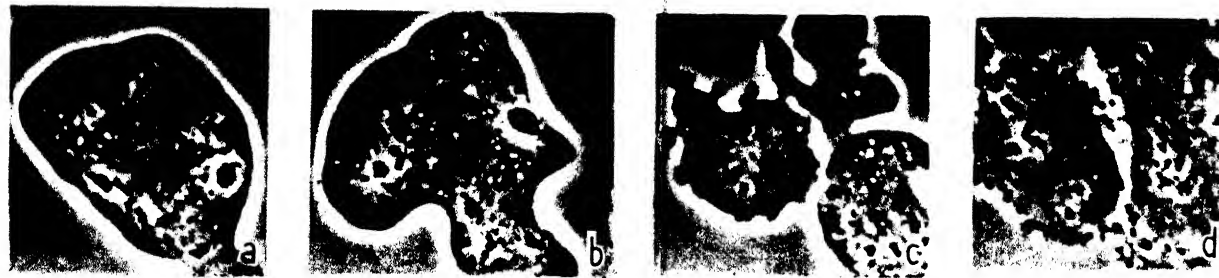
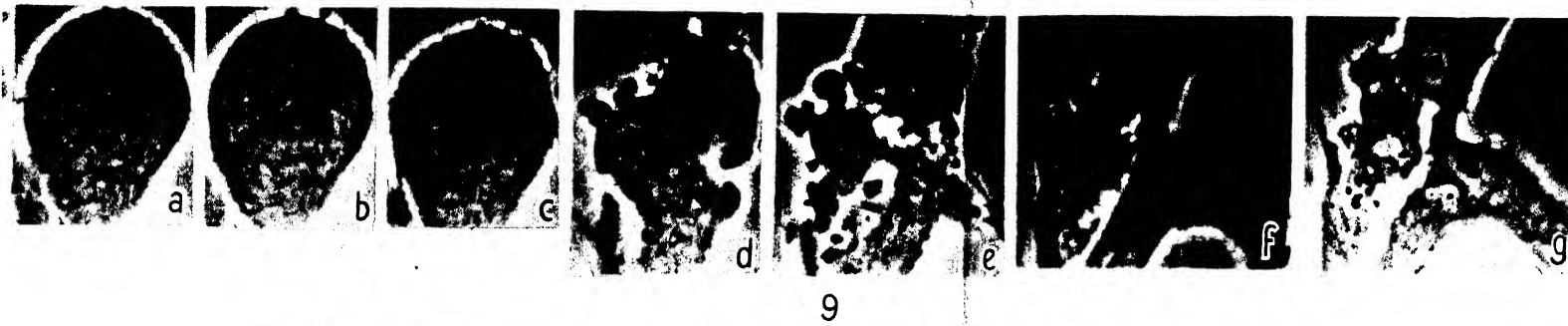
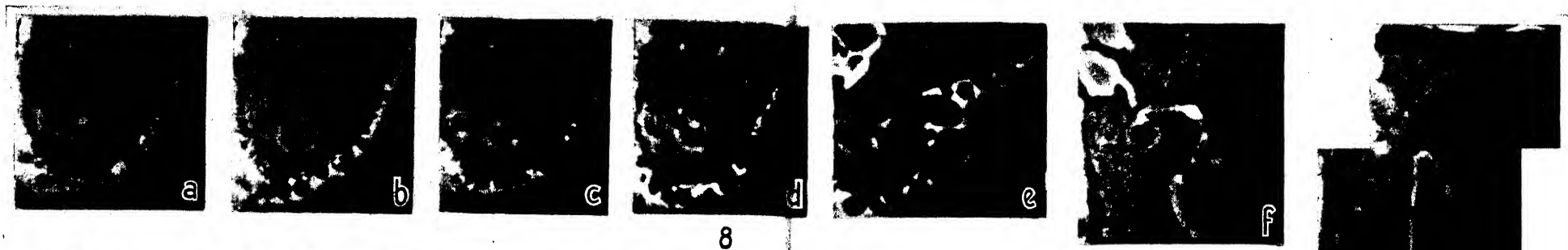
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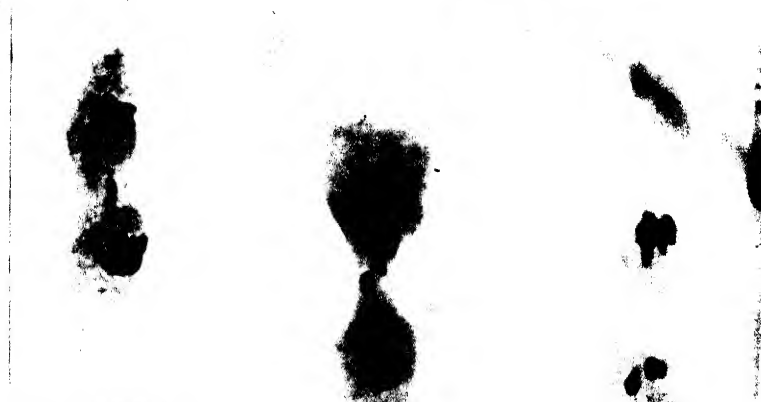


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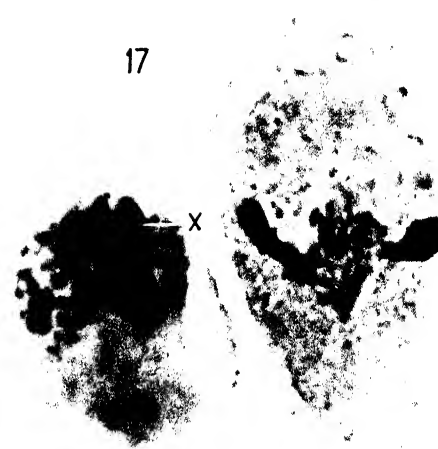
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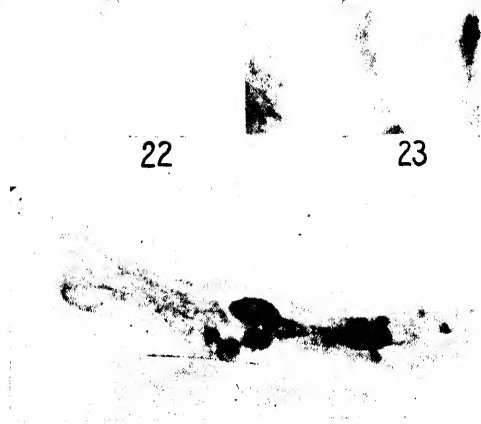


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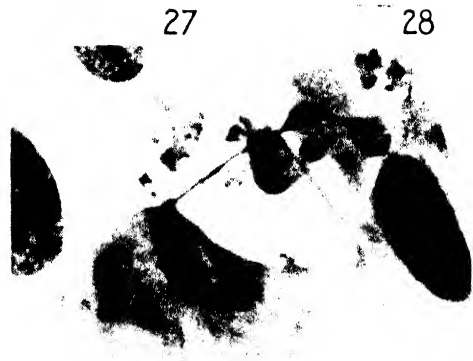


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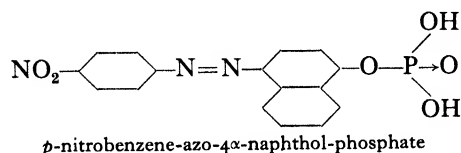
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4. The ester itself shall be at least moderately soluble.
5. The ester shall be stable in the absence of the enzyme.
6. Neither ester nor hydrolysis-products shall be highly inhibitory to the enzyme.
7. The rate of enzyme action on the ester shall be high.

Some of these requirements are not readily compatible with one another, but from work reported previously (Danielli, 1946) it seemed probable that *p*-nitrobenzene-azo-4 α -naphthol-phosphate would comply with many of these conditions.



The phenolic component is, of course, highly coloured. Owing to the presence of a large non-polar moiety and a small polar moiety, it is also highly insoluble in water and alcohol. The ester was expected to be moderately soluble, since the phosphoric acid grouping makes one end of the molecule highly polar and it is known that molecules with a high degree of polar-nonpolar dissymmetry are usually fairly soluble. The reason for this is that the substances form colloidal solutions with the non-polar residues of the molecules lying in the interior of micelles and polar groups at the micelle-water interface. Such solutions should thus contain a low concentration of single molecules of the ester in equilibrium with molecules in micelles. It is doubtful whether an enzyme could act upon the latter, but their presence ensures the maintenance of a supply of single molecules throughout the course of enzyme action.

The chief difficulty anticipated with this ester was that a solution would have detergent properties which might prevent the precipitation of the phenolic component liberated by enzyme action.

PART I. PREPARATION OF *p*-NITROBENZENE-AZO-4 α -NAPHTHOL-PHOSPHATE

Starting from *p*-nitraniline, the ester was synthesized as follows:

1. Diazotization by the 'indirect method' of Saunders (1936), viz. by pouring an aqueous paste of *p*-nitraniline and its equivalent of sodium nitrite on to concentrated hydrochloric acid and ice. The solution was filtered after a few minutes' stirring.

2. Coupling with α -naphthol by stirring the equivalent of α -naphthol in caustic soda solution into the filtered diazotate, with cooling. The *p*-nitrobenzene-azo-4 α -naphthol precipitate was washed with water, alcohol, and ether, and dried, but not recrystallized. (It is recrystallizable with difficulty owing to low solubility in the usual solvents.)

3. Phosphorylation by the method of King and Nicholson (1939), viz. treatment with a slight excess of phosphorus oxychloride in dry pyridine.

Phosphorylation was allowed to proceed for an hour with periodic shaking and the resulting phosphoryl compound decomposed with water.

Hereafter two methods were adopted to obtain a solution of the sodium salt of the phosphoric ester. The solutions obtained by the two methods showed different properties as substrates, as is indicated later.

First solution

The phosphorylated product was diluted with five times its volume of water, throwing most of the unchanged phenol (the phosphorylation does not go to completion) and a part of the ester out of solution. The precipitate was filtered and washed with water to remove pyridine and phosphoric acid. After drying it was digested overnight at 60° C. with 0.4 per cent. sodium veronal adjusted to pH 9.3 with 5N caustic soda. The material was extracted with successive portions of sodium veronal in this way until nothing further was dissolved (as indicated by colour), and the extracts combined and filtered.

The solution, hereinafter referred to as *acid preparation*, had a moderately intense orange colour and showed two components on paper chromatography, viz. an orange band corresponding to the ester and a blue band corresponding to the alkaline (soluble) form of the unchanged phenol. The yield was not good and this may be attributed to two factors: (1) loss of ester during washing of precipitate by solution, (2) rapid acid hydrolysis of the ester immediately upon addition of water after phosphorylation, owing to liberation of phosphoric acid.

Second solution

Saturated baryta was added to the solution after phosphorylation until the solution became strongly alkaline. The precipitated barium salt of the ester was washed exhaustively with water to remove phenol, until the filtrate was no longer blue. It was then washed with 10 per cent. acetic acid to remove barium phosphate. After a final wash with water the barium salt was dried, and an aliquot digested with its equivalent of sodium sulphate in 0.4 per cent. sodium veronal at 60° C. The barium salt of the ester being more soluble than barium sulphate at 60° C., the latter was precipitated and the sodium salt went into solution. The sodium salt itself proved not very soluble and it was necessary to extract the solid material repeatedly with 0.4 per cent. sodium veronal before all was brought into solution. The material thus obtained is referred to hereinafter as *barium preparation*: it had a deep-red coloration, revealed only an orange component on chromatography, and showed a marked detergent activity.

Attempts to obtain the pure dry sodium salt were abandoned in face of three difficulties: (a) low solubility of salt and free ester in the commoner solvents, (b) difficulty of concentrating solutions because of foaming, and (c) tendency of the ester to hydrolyse on prolonged heating of solutions. The concentrations of the solutions employed in these experiments are therefore

defined by preparation and the following table gives quantities employed and yields obtained:

Quantities of Reagents employed in the Several Stages of Preparation of p-nitrobenzene-azo-4 α -naphthol-phosphate

Stages I-3

p-nitraniline	18 gm.	} in 35 ml. water
Sodium nitrite	10 gm.	
Ice	40 gm.	
Hydrochloric acid	40 ml. conc.	
α -naphthol	23 gm.	} in 400 ml. water
Caustic soda	9 gm.	
Yield of p-nitrobenzene-azo-4 α -naphthol	39 gm. (theoretical 44)	

	<i>Acid preparation</i>	<i>Barium preparation</i>
The phenol	5 gm.	5 gm.
Pyridine	15 ml.	15 ml.
Phosphorous oxychloride	2 ml.	2 ml.
Water	100 ml.	2 ml.
Baryta (Ba(OH) ₂ .8H ₂ O)	9 gm. in water
Yield of dry precipitate	5.5 gm.	8.7 gm.
'Free ester' (+phenol)	1 gm.	..
Dry Ba salt	2 gm.
0.4 per cent. sodium veronal	500 ml.	2 litres

The yield of solution given in the barium preparation was double that obtained in the 'acid' preparation, and as judged by colour, about four times as strong. However, even supposing a 100 per cent. conversion to the sodium salt, the final solution was less than 0.1 per cent.

PART II. STUDIES WITH RAT KIDNEY

Preparation of Material

Rat kidney was fixed in thin slices with 80 per cent. alcohol, dehydrated in the usual way, and passed to 54° m.p. wax via methyl benzoate and benzene. Sections were cut at 10 μ and dried on to non-albuminized slides *in vacuo*. After removal of wax, sections were coated with a thin film of celloidin, by passing through a 0.1 per cent. solution before bringing down to water.

Incubation with Substrate

Sections were incubated at 37° C. in the solutions prepared as above to investigate the influence upon enzymic action of the following factors:

- (i) Time of incubation.
- (ii) Degree of dilution of substrate (all dilutions were made with 0.4 per cent. sodium veronal).
- (iii) pH of solution (adjusted by addition of 5N caustic soda or hydrochloric acid).

Results

In these initial experiments no enzyme action was observed using the barium preparation (but see later). When the acid preparation was used, *p*-nitrobenzene-azo-4 α -naphthol was deposited in the brush border and lumina of the proximal convoluted tubules, in most of the nuclei of the tubule epithelium, in nuclei in the glomeruli, and around the adventitia of arterioles—i.e. in all the sites which give a positive reaction with Gomori's technique. The nuclear reaction, however, was far more intense than at other sites and than that shown by Gomori's method, the structure of the nuclei in most cases being completely obliterated by deposits of dye (Pl. I, fig. 1).

Optimal results were given by the strongest solution, 1 hour's incubation being adequate. The substrate was acted upon over a pH range of 7.0–9.5, showing an optimum in the region of 8.7. Variation of pH, time, or dilution did not appear to alter the relative activity of nuclear and extranuclear enzyme. The tissue generally took up unchanged substrate and became yellow stained. This background staining was quickly removed in 50 per cent. alcohol. Most preparations were mounted in glycerine jelly for inspection, but very good mounts were obtained in balsam so long as dehydration and clearing were carried out rapidly. (The dye is slightly soluble in xylene.) Balsam mounts had the advantage of complete absence of background staining.

Further Experiments with the Barium Preparation

Since the enzyme hydrolysed the ester in the 'acid preparation', the question arose as to why it was inactive towards the 'barium preparation'. The only likely impurity in this preparation was sodium sulphate in excess, but this was shown not to inhibit the enzyme when used in Takamatsu and Gomori's incubation mixture. The hypothesis was adopted that, when this substrate is employed, the enzyme cannot act in the total absence of its end-products, since it was known that the acid preparation was contaminated with at least the phenolic component. (It probably also contained phosphate since adequate washing of the phosphorylated product was very difficult to achieve.) A series of solutions was accordingly partially hydrolysed by bringing to pH 2 at 60° for varying times, and kidney sections incubated in these after cooling and readjustment of pH. It was found that only momentary acid treatment was necessary to render the substrate available to the enzyme, but 30 minutes' prehydrolysis was optimal. Brush border activity was very good with these solutions, but nuclear phosphatase gave a far less intense reaction than with the acid preparation. The degree to which hydrolysis had proceeded was checked roughly by paper chromatography of an alkaline solution, and the general indication was that, within limits, the nuclear reaction was more marked the farther preliminary hydrolysis had proceeded (Pl. I, figs. 2 and 3).

Prolonged incubation, i.e. for more than 12 hours, in a non-prehydrolysed solution, gave a heavy deposit of dye in the tubules but no visible nuclear reaction (Pl. I, fig. 4).

From these experiments it seemed probable that the hydrolysis products of the ester were in fact activators of the enzyme. To confirm this, sections were incubated in solutions to which had been added phosphate or the phenol. It was found that as little as 0.1 mg./100 ml. of disodium phosphate was sufficient to instigate enzymic hydrolysis. The picture given was similar to that by Gomori's method, i.e. good brush border deposits but only a moderate nuclear reaction (Pl. I, fig. 5). It was found difficult to bring the phenolic component into solution at pH 9 without prolonged warming which would undoubtedly cause partial hydrolysis of the ester. But sections incubated in an 'inactive' solution to which had been added the solid phenol gave, after 2 hours, a strong reaction in many nuclei with no corresponding action in the brush borders. Staining was, however, confined to nuclei in the innermost region of the cortex, which are normally the most reactive (Pl. I, fig. 6).

Lastly, sections were incubated in (a) a solution which had been completely prehydrolysed, (b) an 'inactive' solution, and (c) a mixture of these: only in the third series was a histochemical reaction shown.

Sections which had been immersed in water at 95° C. for 5 minutes gave no reaction with any solution.

Discussion

It seems fairly clear that, *with this substrate*, end-products of the enzymic hydrolysis must be present at least in trace quantities before enzyme action can start. In this connexion it may be recalled that phosphorylases cannot synthesize glycogen from glucose 1-phosphate unless a trace of glycogen be present *ab initio*. This difficulty can be overcome either by preparing the substrate as for the 'acid preparation' above, or by slight prehydrolysis or addition of phosphate to the 'barium preparation'. One of the latter is advised since the preparative procedure is more straightforward and better yields are obtained.

A differential action is shown by nuclear and extra-nuclear enzymes according to the relative amounts of the two end-products, phenolic or phosphate, present. Thus deposition of dye in the brush border appears to be independent of the prior presence of the phenolic component, but is enhanced by phosphate, whereas nuclear activity is enhanced by the presence of the phenol.

PART III. STUDIES WITH CALCIFYING TISSUES

Since one of the main advantages anticipated from this technique, compared with that of Takamatsu and Gomori, was that the presence of preformed insoluble phosphate should not interfere, some tests have been carried out with dogfish and rat material showing calcifying cartilage and bone. This material, kindly supplied by Miss I. J. Lorch, consisted of 8 μ sections prepared in the same manner as the kidney used above, and cut from the following regions:

1. Transverse sections of the branchial region of dogfish embryo.

2. Transverse sections of the orbital region of dogfish embryo.
3. Longitudinal sections of ribs of the adult rat, passing through the chondrocostal junction.

Sections of rat ribs gave a demonstration of phosphatase activity similar to the Gomori method after 2 hours' incubation at pH 8·7; i.e. a good deposit of dye appeared in the inner layer of the periosteum, in the bone-marrow, and at the chondrocostal junction. Dogfish material did not show a great activity by either method. Although tested at several pH values and at 26° or 37° C., our substrate did not give as good a histochemical picture as obtained by Gomori's technique. Enzyme activity was revealed at the following sites in the times of incubation indicated:

- A. Sections in branchial region: between neural arch cartilages: 15 hours.
- B. Orbital region:
 1. Beneath young dermal denticles, especially buccal teeth: 2 hrs.
 2. Perichondrium of Meckel's cartilage and mandibular arch: 15 hrs.
 3. Outer edge of choroid layer of eye: 2 hrs.
 4. Along pia mater: 2 hrs.
 5. In cavity of mid-brain: 2 hrs.

In no case was any nuclear reaction shown even when solutions optimal for nuclear reaction in kidney were used. The Gomori method gave stronger reactions throughout and also showed up other sites of activity, e.g. around sensory mucous canals, not shown by our method. The technique cannot, therefore, be considered suitable for dogfish material except to reveal sites of comparatively great enzyme activity.

The revelation of phosphatase on the choroid layer is interesting, since it is difficult to observe by Takamatsu and Gomori's method owing to heavy black pigmentation already present. Also phosphatase associated with the denticles is difficult to observe by their method since calcium phosphate already present is not removed satisfactorily by decalcification procedures and a 'blank' staining is therefore produced.

A further very useful feature of this substrate revealed by these tests is that it stains calcified regions strongly yellow within 2 hours, presumably owing to formation of the calcium salt of the unchanged ester; this colour is not removed in alcohol or xylene. Thus a picture is obtained in one operation showing calcified regions (stained yellow) and regions of enzyme activity (stained red). Should any doubt arise as to whether the colour is in fact due to the free phenolic dye and not to 'background' or calcium staining, the doubt may be immediately resolved by testing with dilute caustic soda: this causes the phenol to change to deep blue, but is without effect on the ester. This test cannot be carried out on a section which is to be kept, as the blue alkaline form of the phenol rapidly diffuses out of the tissue.

CONCLUSION

The phosphate ester prepared in this work has most of the properties specified in the introduction. The colour intensity is satisfactory. Some attempts were made to intensify it further, by coupling the phenol liberated by phosphatase with diazonium hydroxides, without success: the degree of alkalinity required for the coupling of further diazo residues brings about rapid dissolution of the dye. The solubility of the dye is very low in acid and neutral solution but increases with alkalinity: up to pH 9, however, it is sufficiently insoluble for the purpose required. It is slightly soluble in alcohol and xylene, so that sections containing it must be passed rapidly through these substances if balsam mounts are required. The introduction of a polar group into the molecule, whilst decreasing its solubility in non-polar solvents, would increase the solubility in water and was therefore not deemed advisable. The dye concerned seems to offer a good compromise between solubility in the two types of solvent. Some difficulty has been experienced with recrystallization in the section as a result of prolonged incubation. When this occurs very long wavy needles are formed which might be mistaken for connective tissue fibres or cell processes. Recrystallization is avoided if incubation is not allowed to proceed for more than 3 hours at 37°.

The ester is not very soluble but sufficiently so. Stronger solutions than those used in this work (approx. 0.1 per cent.) would be of no value, however, owing to high detergent power. On the other hand, the failure of phosphatase in certain sites to act visibly on this substrate might be associated with the diluteness of the solution, since elsewhere, e.g. in kidney, the concentration was found to have a marked effect.

The ester is not completely stable in the absence of the enzyme, becoming increasingly rapidly hydrolysed with decrease of pH. No difficulty should be encountered from this source if the procedure as outlined is adopted and solutions are filtered immediately before use. There is some difficulty about point 7, viz. the rate of hydrolysis of the ester. Whilst some of the sites shown to have activity by Takamatsu and Gomori's method show high activity with the present technique, other sites showing only low activity by their method show none with the new ester. Whilst many factors may in fact be involved, the simplest explanation at present available is that there are many alkaline phosphatases, and a given substrate may be rapidly acted upon by some of these, slowly by others, and by some not at all. If this is so, the ratio of activity towards β -glycerophosphate and *p*-nitrobenzene-azo-4 α -naphthol-phosphate is not the same for all alkaline phosphatases.

Our opinion is that for general use the new substrate is not as satisfactory as β -glycerophosphate. The sharpness of cytochemical localization is not as great as that obtained with glycerophosphate, and often sites of low activity for β -glycerophosphate appear to have no activity with the new substrate—though occasionally this relationship is reversed. The new method is particu-

larly valuable for pigmented tissues, and for tissues containing preformed calcium phosphate.

In our studies with this new reagent, it was observed with tissue cultures that if a site did not display some phosphatase activity after 4 hours, it would not display activity after even 24 hours. Similar behaviour has been found with the technique of Takamatsu and Gomori, except that the critical period is of the order of 12 hours. With longer incubation all that happens, with both techniques, is an increase in the intensity of reaction in sites which have already displayed activity. We believe this phenomenon to be caused by a physico-chemical limitation which must be common to all techniques involving precipitation of a reaction product, the principle of which is as follows: the formation of a precipitate is dependent upon a threshold number of molecules becoming present simultaneously in a very small unit of volume. Unless this minimum or threshold is reached, precipitation will not occur, even though the solution is theoretically saturated, and the molecules concerned will diffuse until a suitable crystallization centre is reached. Thus a site of enzyme activity can only be revealed if the enzyme activity is sufficient for the threshold condition to be reached—sites of lower activity will always appear negative. Consequently with this type of technique negative results after very prolonged incubation periods do not necessarily indicate complete absence of enzyme, but possibly only that the enzyme activity is below threshold for the method in question.

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SUMMARY

1. A new method is described for the cyto- and histochemical demonstration of alkaline phosphatase (monoesterase), a synthetic substrate *p*-nitrobenzene-azo-4 α -naphthol-phosphate being employed as the sodium salt. Two methods for preparing a solution of this substance are described, and optimal conditions for enzymic hydrolysis of the ester worked out by the use of kidney sections.

2. Experiments are also described in which the substrate has been used to demonstrate phosphatase activity in calcifying tissues of dogfish and rat.

3. The results of the experiments with rat kidney indicate that traces of end-products are necessary before the enzyme can attack this substrate.

4. When results obtained with the new substrate are compared with those obtained with β -glycerophosphate, it is found that some sites display more activity towards one substrate than to the other.

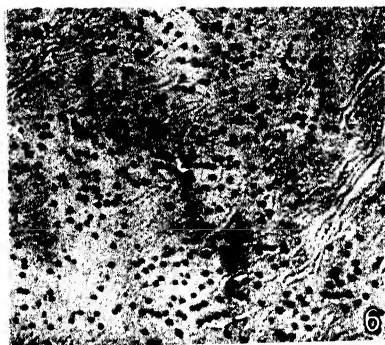
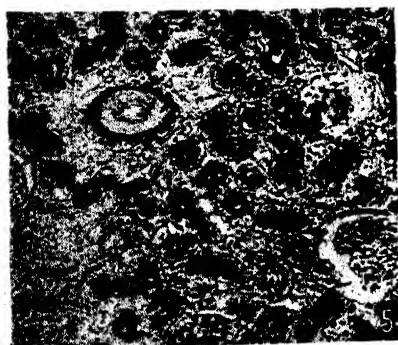
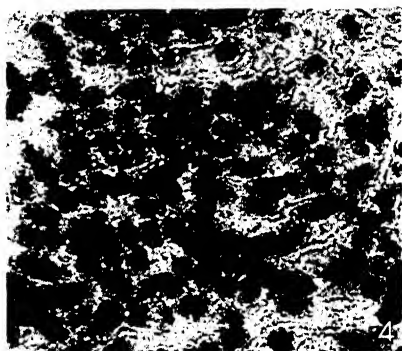
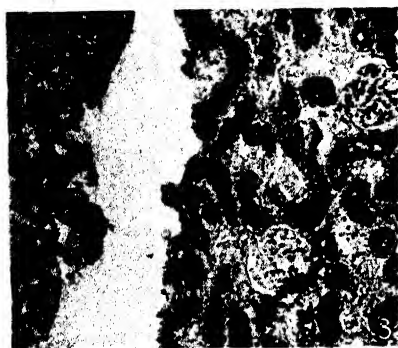
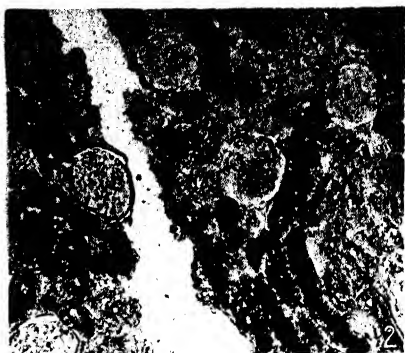
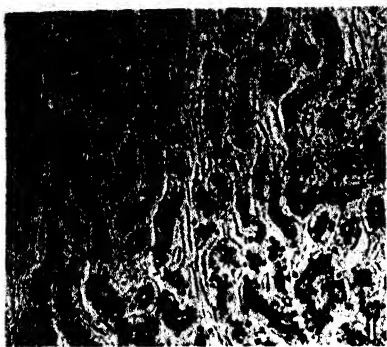
5. The cytochemical localization of phosphatase with the new substrate is not as precise as with β -glycerophosphate.

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EXPLANATION OF PLATE I

- FIG. 1. Rat kidney incubated 1 hr. in undiluted 'acid preparation' showing strong brush border and nuclear deposit.
 FIG. 2. Rat kidney incubated 1 hr. in half-strength 'barium preparation' after momentary prehydrolysis at pH 2.
 FIG. 3. Rat kidney incubated 1 hr. in half-strength 'barium preparation' after 30 minutes' prehydrolysis.
 FIG. 4. Rat kidney incubated 15 hrs. in undiluted 'barium preparation' without hydrolysis or addition of end products.
 FIG. 5. Rat kidney incubated 1 hr. in half-strength 'barium preparation' containing 1 mg. disodium phosphate /100 ml.
 FIG. 6. Rat kidney incubated 2 hrs. in half-strength 'barium preparation' containing added *p*-nitrobenzene-azo-4 α -naphthol.



A. LOVELESS AND J. F. DANIELLI.—PLATE I

A Critical Study of Techniques for the Cytochemical Demonstration of Aldehydes

BY

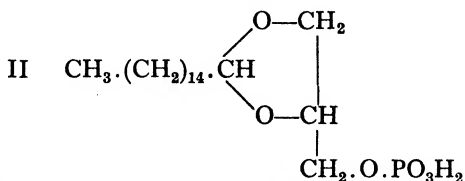
J. F. DANIELLI

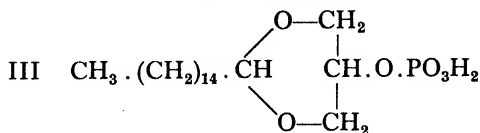
(From the Chester Beatty Research Institute, The Royal Cancer Hospital, London, S.W. 3.)

I. INTRODUCTION

ALDEHYDES, as was shown by Feulgen and Voit (1924), can in principle be demonstrated by treatment with reduced fuchsin. The aldehyde and the reduced dye combine, as in the familiar Feulgen reaction, to give a coloured product. It is clear, however, that if the aldehyde, or the product of its reaction with reduced fuchsin, is diffusible, the results obtained would be worthless from a cytological point of view. Aldehydes can be profitably studied with the aid of existing techniques only if (a) the aldehydes are themselves indiffusible, and (b) if their reaction products with reduced fuchsin are indiffusible. Aldehyde groups having these properties may form part of large colloidal molecules such as proteins and nucleic acids, and also occur as long-chain aliphatic aldehydes such as palmital (see I, below) and stearyl (corresponding to palmitic and stearic acids). Aldehydes belonging to the protein-nucleic acid group may be indiffusible in both aqueous and organic solvents. The long-chain aliphatic aldehydes are indiffusible in aqueous media, but diffusible in organic media. Consequently the aliphatic aldehydes, which have been the main group of compounds studied in this work, are the more difficult group to investigate.

The aliphatic aldehydes are only partially present in tissues in the free state. Feulgen and Bersin (1939) have shown that the remaining part is present in an unstable complex, from which the aldehyde may be liberated by brief treatment with cold N/10 hydrochloric acid or with saturated mercuric chloride solution. These authors have also presented strong evidence that the complexes are the acetals of the aldehydes formed with glycerophosphate, with structures II and III.





It is well known that aldehydes are readily liberated from acetals by dilute acid, but it is not altogether clear why mercuric chloride should also bring about this transformation. Furthermore, there is a possibility that mercuric chloride oxidizes compounds containing groups such as $-\text{CO} \cdot \text{CHOH}-$ and $-\text{CO} \cdot \text{CHNH}_2-$ to aldehydes, so that mercuric chloride must be used with discrimination. In Section 7 of this paper, a procedure is given which distinguishes between (a) free aldehyde groups, (b) aldehyde groups bound as acetals, and (c) aldehyde groups liberated by oxidation.

The investigation reported here was made on mouse and rat liver. It was planned to secure answers to the following four questions:

- (i) Is the aldehyde in a fixed preparation in its normal physiological position?
- (ii) Is the method specific for aldehydes?
- (iii) How much aldehyde is destroyed by the procedure?
- (iv) Does the colour developed by the technique appear at the site of aldehyde in the fixed preparation, or at sites having a high affinity for the coloured reaction product?

2. NORMAL PROCEDURE

The procedure for demonstrating aldehydes by the technique of Feulgen and Bersin has been found to give optimal results when used in the following manner:

- (a) Pieces of tissue not more than 2 mm. thick are fixed in a solution containing 8 per cent. formaldehyde and 5 per cent. acetic acid, or 8 per cent. neutral formaldehyde.
- (b) After material has been fixed for not less than 2 hours and not more than 5 days, sections are cut on a freezing microtome.
- (c) The sections are washed with distilled water and then allowed to stand in saturated mercuric chloride solution or cold N/10 hydrochloric acid for 15 minutes.
- (d) The mercuric chloride or hydrochloric acid solution is sucked off and the sections are washed twice with distilled water.
- (e) Sections are allowed to stand in reduced fuchsin solution for 15 minutes.
- (f) Fuchsin solution is sucked off and the sections washed with a solution containing SO_2 . Three washes each of 5 minutes duration are used. SO_2 solution is made by mixing 10 ml. of 10 per cent. sodium bisulphite solution with 10 ml. of normal hydrochloric acid, and diluting to 200 ml.
- (g) Sections are washed with distilled water and mounted either in neutral balsam or glycerine jelly.

Where sections are robust, they may be shaken during the washings on a mechanical shaker. Otherwise an occasional agitation by hand is necessary to secure adequate penetration of the sections by the various reagents. The purpose of washing with N/10 hydrochloric acid solution is to liberate aldehyde from acetals such as II and III above. Washing with mercuric chloride solution has also been stated to release aldehyde from acetals: in addition it



FIG. 1.

may form aldehyde groups by oxidation of compounds containing groups such as $\text{—CO}\cdot\text{CHOH—}$. During step (e) some of the reduced fuchsin usually suffers atmospheric oxidation, and the oxidized dye stains the sections in a non-specific manner. This non-specific stain is removed by the washing with SO_2 solution. Text-fig. 1 shows the characteristic appearance of a rat-liver section treated by this procedure.

3. OTHER FIXATIVES

A considerable variety of fixatives was tried. Some were acid, some neutral, some alkaline, and some contained in addition mercury or formaldehyde.

Furthermore, the effect was tried of pre-precipitation of the material in the tissue by the use of saturated solutions of calcium chloride, **magnesium sulphate**, or ammonium sulphate. The intensity of the aldehyde reaction, and the nature of its distribution in liver sections, was found to be independent of the physical nature of the fixative. It is therefore clear that the aldehyde revealed by this technique must be occupying its normal physiological position, unless the colour developed appears at a site having a high affinity for the reaction product of reduced fuchsin with aldehyde, and not at the original site of the aldehyde. This latter contingency is an unlikely one, for there is no dependence of the distribution of aldehyde upon the nature of the fixative. Experiments will be described later which clarify the position more precisely.

The fixative components which were tried were formaldehyde, acetic acid, mercuric chloride, pyridine, and trichloroacetic acid. The only mixtures of these substances which were found unsatisfactory were those containing trichloroacetic acid: after fixation in mixtures containing trichloroacetic acid, nuclei frequently stain with reduced fuchsin, without further treatment. It is of course impossible in this technique to use fixatives containing a high concentration of an organic liquid such as alcohol or acetone because the aliphatic aldehydes are soluble in these solvents.

The procedure could be greatly simplified if it were possible to fix tissue in a fixative containing both mercury and reduced fuchsin. Of course formaldehyde could not be used in such a fixative. But so far no success has been obtained with mercury-reduced fuchsin mixtures—possibly because of poor penetration.

The choice of 5 per cent. acetic acid, 8 per cent. formaldehyde as a fixative for general work was based on the facts that the fixation obtained is relatively good, both for the cytoplasm and for the nucleus, and that the formaldehyde present protects the tissue aldehydes from being oxidized by atmospheric oxygen. This fixative was used in all the experiments reported here, unless otherwise indicated. Formaldehyde present in the tissue sections must of course be removed before the section reaches the reduced fuchsin solution. This end is sufficiently procured by the procedure described above. It is not, however, possible to keep material for an unrestricted period of time in this fixative, for polymerization products of formaldehyde are slowly deposited in the fixed material. These polymerization products can also give a colour with reduced fuchsin, which tends to mask the colour developed by the naturally occurring aldehydes. Where it is desired to observe free aldehyde groups only (i.e. not acetal aldehyde), fixation is preferably in 8 per cent. neutral formol.

Some material (e.g. Rous tumour) after fixation in acetic-formaldehyde gives frozen sections which are sticky and difficult to handle. This material can be brought under control by the following procedure: after fixation for at least 2 hours in 5 per cent. acetic acid+8 per cent. formaldehyde, the pieces are transferred to a Thunberg-tube containing 4 per cent. gelatin at 37° C. to which has been added, just prior to the transfer, 40 per cent. formaldehyde

solution to bring the gelatin up to a content of 4 per cent. formaldehyde. The tube is evacuated and kept at 37° C. for 1 hour, then cooled in ice-water. Frozen sections may be cut as soon as the gelatin has set. No significant destruction of tissue aldehyde is caused by this procedure.

4. TESTS FOR THE SPECIFICITY OF THE REACTION

From the studies of the reactions of aldehydes with reduced fuchsin which have been made by other workers, it seems unlikely that groups other than the aldehyde group CHO can react with reduced fuchsin to give the characteristic purple colour. But to guard against the possibility of other substances being involved, a number of supplementary techniques have been developed.

(a) That the substance involved is fatty in character can be shown by extracting sections with fat solvents between steps (b) and (e) of the procedure given in Section 2 of this paper. Washing may be carried out with alcohol-ether mixtures followed by xylene, or by acetone only. The extraction should be carried out for at least 15 minutes, during which three changes are made of the extracting fluid. The aldehyde is almost entirely removed by extraction.

(b) A characteristic reaction of aldehydes is the development of a purple colour with azobenzene phenylhydrazine sulphonic acid. The procedure is as follows: sections are taken through the procedure given in Section 2 of this paper up to step (d). Individual sections are then removed from the bath and placed in a solution composed of 7 drops of a saturated aqueous solution of the azobenzene phenylhydrazine sulphonic acid and 4 drops of concentrated sulphuric acid in the bottom of a test-tube. This is heated in a boiling-water bath for 30 seconds, after which a large excess of water is added. The sections are then mounted on a slide in concentrated hydrochloric acid. Under these conditions the aldehydes form a compound with azobenzene phenylhydrazine which is purple. The distribution of the purple colour so found is similar to the distribution of the colour found with Feulgen's method. Although the distribution of the colour is the same as that found in Feulgen's method, the technique is not suitable for general use because fairly rapid hydrolysis of the tissue takes place in the strong acid which is used as a mounting medium. If not mounted in strong acid, the aldehyde is coloured a pale yellow only, which is unsatisfactory for general purposes.

(c) A characteristic reaction of aldehydes is their ability to reduce ammoniacal silver nitrate, giving a black precipitate. The silver solution is prepared by adding concentrated ammonia solution to 5 per cent. silver nitrate solution in water, until the precipitate which forms at first is completely redissolved. Sections are placed in this solution and left for 2 hours at 60° C. in the dark. The distribution of the black precipitate so obtained in liver sections, taken after step (d) of the procedure given in Section 2 of this paper, is similar to the distribution of colour obtained with the reduced fuchsin method.

(d) A characteristic reaction of aldehydes is the formation of a yellow hydrazone with 2:4 dinitrophenylhydrazine. A saturated solution of 2:4 dinitrophenylhydrazine in N hydrochloric acid is cooled to 0° C. Sections

from step 2(d) are placed in this solution and left for either 2 hours or 20 hours. They are then washed quickly in cold N hydrochloric acid and mounted in glycerine jelly. In liver sections the distribution of colour so obtained is similar to that obtained with the reduced fuchsin method.

(e) A characteristic reaction of aldehydes is their ability to form oximes by combination with hydroxylamine. If, therefore, the purple colour formed with reduced fuchsin is due to combination of the reduced fuchsin with aldehyde groups, then prior treatment with hydroxylamine should eliminate the purple colour. The procedure is as follows:

2 gm. of solid hydroxylamine hydrochloride and 4 gm. of sodium acetate are dissolved in 6 ml. of distilled water. Sections are taken through the procedure given in Section 2 of this paper for steps (a) to (d) inclusive, and then are placed in the hydroxylamine solution at room temperature, and left for 1 hour. The sections are washed in distilled water and treated with the reduced fuchsin solution, &c., as in steps (e) to (g) of the procedure in Section 2. When liver sections are treated in this way, the appearance of colour with reduced fuchsin is entirely eliminated.

These five supplementary tests prove conclusively that the colour obtained with reduced fuchsin in liver sections is due to the presence in the sections of aldehydes. The solubility of the aldehydes in lipid solvents shows that these aldehydes are fatty in character. These tests may of course be applied to practically any other tissue.

5. THE DEGREE OF DESTRUCTION OF ALDEHYDE

The possibility exists that aldehyde is partly destroyed by the procedure. This has been tested by increasing the duration of each step in turn in the routine by a factor of fourfold. This can be done without causing any significant loss of the colour formed by reduced fuchsin in the case of liver sections. It is therefore clear that no substantial loss of aldehyde is caused by any of the steps in the procedure. It is, however, necessary that step 2(a) should not occupy more than 5 days for the best results. Keeping tissue for longer periods than this in the formaldehyde-acetic mixture may result in the deposition of other aldehydic material in the tissue; and if the tissue is removed from formaldehyde, the naturally occurring aldehydes in the tissue tend to be lost by oxidation.

6. THE RELATIONSHIP BETWEEN THE SITE OF ALDEHYDES AND THE POSITION OF THE COLOUR IN THE FUCHSIN REACTION

The possibility exists that the purple colour obtained with the reduced fuchsin reagent diffuses to regions which have a high affinity for this coloured substance. If this is so, the results obtained will not show the original site of the aldehyde. It has been pointed out, in Section 3, that this is an unlikely contingency. It is made still more unlikely by the fact that the distribution of colours in Tests 4(b), (c), and (d) is the same as that of the fuchsin. Furthermore, the localization of the purple colour may be very sharp. Occasionally in

the liver one intensely stained cell will be seen, completely surrounded by quite unstained cells, and in this cell the greater part of the aldehyde may be sharply localized in one part of the cytoplasm. The nucleus is practically always unstained.

However, the position can be made more secure by getting the aldehydes into a diffusible condition, combining them with reduced fuchsin, and then placing sections from which all aldehyde has been removed in this mixture. The positions in which colour is taken up will be those which have a high affinity for the aldehyde-fuchsin compound. The procedure used for this was to extract rat-liver with alcohol and then with xylene, in an atmosphere of nitrogen. The alcohol and xylene were boiled off *in vacuo* and the residue dissolved in a small volume of alcohol. When this alcoholic solution was poured into water it gave a colloidal solution. To this was added reduced fuchsin, whereupon a purple colour appeared almost immediately. Into this solution were placed sections from which all the aldehyde had been removed by extraction, as in procedure 4(a). After 15 minutes the sections were removed and washed in SO_2 solution. It was found that the nuclei were intensely stained and there was a uniform diffuse staining of the cytoplasm. There was no highly localized staining of the cytoplasm. This distribution of colour is quite different from that found with the procedure given in Section 2.

One can therefore conclude that the purple colour does in fact appear at the site of aldehyde occurrence. If the aldehyde were able to diffuse, a quite different cytological picture would be obtained from that given by the procedure given in Section 2.

7. DIFFERENTIATION BETWEEN FREE ALDEHYDE, ALDEHYDE ACETAL, AND ALDEHYDE LIBERATED BY OXIDATION

According to Wislocki and Dempsey, aldehydes may be formed from α -hydroxy ketones by cold aqueous mercuric chloride. Boscott, Mandl, Danielli, and Shoppee (1948) have demonstrated that this is not so with typical α -hydroxy ketones, such as deoxycorticosterone. Nevertheless, it is conceivable that atypical compounds would be oxidized to aldehydes under these conditions. Thus it may be necessary at times to discriminate between (i) free aldehydes, (ii) aldehydes present as acetals, and (iii) aldehydes liberated by oxidation. This can be done as follows, after fixation with neutral formaldehyde solution:

- (i) Expose to reduced fuchsin, omitting treatment with mercuric chloride or hydrochloric acid in steps (c) and (d) of the general procedure of Section 2. This demonstrates free aldehyde only. Care must be taken to avoid using an acidic fuchsin solution.
- (ii) Use of N/10 hydrochloric acid in steps (c) and (d) will show both free and acetal aldehyde. But if the free aldehyde is converted to oxime, as in procedure 5(e), before steps (c) and (d), only acetal aldehyde will be demonstrated.
- (iii) Use of mercuric chloride solution in steps (c) and (d) may show, in

addition to free aldehyde and acetal aldehyde, aldehyde formed by oxidation. Treatment with cold N/10 HCl, followed by oxime formation, then by mercury treatment, shows only aldehyde formed by oxidation.

8. DISCUSSION

In the introduction to this paper it was pointed out that satisfactory answers to four questions must be obtained before the reduced fuchsin method for demonstration of aldehydes could be regarded as satisfactory. The experiments described in Sections 2 to 6 of this paper define conditions under which the procedure may be carried out, so as to obtain results which comply with the restrictions and requirements of these four questions. The supplementary procedures 4(b), (c), and (d) are not as satisfactory as the reaction with reduced fuchsin. But they are very necessary supplementary techniques for making certain that the colour developed is in fact due to aldehyde groups. As a routine check that the purple colour formed after treatment with reduced fuchsin is indeed caused by the presence of aldehydes in the material, it is best to use test 4(e). With this test all colour caused by presence of aldehydes is eliminated by formation of the aldoxime before exposure to reduced fuchsin.

The chemical specificity of the reaction with reduced fuchsin for aldehydes has been examined on the test-tube scale by a number of other workers, particularly by Oster and Oster (1946). The work of Oster and Oster shows that none of the other compounds likely to occur in tissue sections will in fact give a purple colour under the conditions they use. The main possible source of error lies in the resemblance between ketones and aldehydes. But the experiments of Oster and Oster seem to make it clear that none of the known ketones will in fact react with reduced fuchsin. There is, however, clearly a need for a reaction which is specific for keto groups.

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SUMMARY

A critical study has been made of Feulgen's technique for demonstrating tissue aldehydes. Conditions are given within which the technique is reliable on the cytological scale. Five supplementary tests for aldehydes are described. It is shown how distinction may be made between free aldehyde, acetal aldehyde, and aldehyde formed by oxidation.

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On the Significance of the Plasmal Reaction

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INTRODUCTION

THE 'plasmal reaction' has been widely used in cytology, and is generally interpreted as revealing the presence of aldehydes, but there is considerable doubt as to their source. According to Feulgen and his collaborators, who first described the reaction, the aldehydes (plasmal) are formed by decomposition of acetalphosphatides (plasmalogen) under the catalytic influence of mercuric chloride. According to Verne, they are formed by oxidation at unsaturated linkages in various forms of lipid, the explanation of the action of mercuric chloride being either that it 'unmasks' the lipoids (Gérard) or that it destroys the inhibiting effect produced by formaldehyde when that substance is used in fixation.

The techniques used for demonstrating the plasmal reaction vary considerably, having in common only the use of Schiff's reagent and the production of results in the cytoplasm and its inclusions, not in the nucleus.

This paper presents evidence to show that the variations in technique are of importance and affect the results. There is no evidence that aldehydes are concerned in the reaction as practised by cytologists, but there is good evidence that oxides and hydroperoxides are the groups responsible in many cases, and that although acetal linkages will give rise to aldehydes under the conditions of the improved technique suggested, there is no *histochemical* evidence that such linkages are in fact present in the tissues investigated. Such substances have been prepared from tissues by Feulgen and his school, but the plasmal technique as usually practised does not show them clearly.

COMPARISON OF TECHNIQUES

The work of Feulgen and his school has been reviewed from the biochemical standpoint by Feulgen and Bersin (1939) and completed by the synthesis of an acetalphosphatide (Bersin *et al.*, 1941). Imhäuser (1927) carried out a [Quarterly Journal Microscopical Science, Vol. 90, part 1, March 1949]

survey of the occurrence of plasmal in tissues, and concluded that it was very widespread indeed. The technique was described by him and by Feulgen and Voit (1924).

Verne has been the principal investigator of plasmal and has modified the technique. He considers that positive results are due to the production of aldehydes by the atmospheric oxidation of double bonds (1928a, b, c, 1929a, b, 1936a, b, 1937a, c). Lison (1932) showed that Schiff's reagent would react with oleic acid and certain other unsaturated substances besides aldehydes. He noticed that not all of these would react with other compounds used to characterize aldehydes, nor did a reaction with one of these compounds mean that the substance would react with others. Oxidant enzymes might also recolour Schiff's reagent. Gérard (1935) believed that, myelin excepted, a positive result was always due to 'oxidases'. Lison (1936a) showed that weak positive results with the Nadi reagent were due to peroxides formed in lipoids by atmospheric oxidation. Verne (1937b, 1940) disagreed with these conclusions and reaffirmed his theory that aldehydes were responsible.

The techniques used were as follows. All employ sections cut on the freezing microtome.

1. *Technique of the Feulgen School*

Frozen sections of fresh tissue are used, some being treated with mercuric chloride solution, the rest acting as controls. Untreated sections negative or only weakly positive with Schiff's reagent, treated sections intensely positive.

Since this is the technique prescribed by Feulgen, it alone can be called the plasmal technique.

2. *Technique of Verne*

Tissues are fixed in a mercuric chloride or platinum chloride fixative. No control section.

Lison (1933) referred to this as the Feulgen-Verne technique (F.V.), a name usually employed by Verne himself.

3. *Technique of Gérard*

Tissues are fixed in a formaldehyde-fixative which is carefully washed out. A control section is used, which is left in distilled water while its fellow is in mercuric chloride solution.

4. *Technique of Guyon (1932)*

This appears to be the plasmal technique as applied to myelin. Both Guyon and Verne refer to positive results with fresh tissue. This does not, apparently, mean that fresh tissue will recolorize Schiff's reagent. A short treatment with mercuric chloride is necessary. However, Verne (1937a, p. 4) does seem to refer to a positive result without mercuric chloride.

Most investigators have used preliminary treatments with phenylhydrazine or semicarbazide or their derivatives to prevent the reaction. Sodium

bisulphite and dimedone have also been employed. But these will react with compounds other than aldehydes, and most are strong reducing agents which might be expected to prevent atmospheric oxidations. Their value in histochemistry is discussed below (p. 82).

EXPERIMENTAL RESULTS

The following questions arise from the preceding discussion:

What is the relation between the results obtained by the various techniques?

How far can the phenomena observed be reproduced with unsaturated lipoids *in vitro*?

Is the technique entitled to rank as a histochemical test?

They will be dealt with in the order given.

A. *The Relations between the Various Techniques proposed*

Feulgen's original technique—the plasmal technique as it will be called in this paper—was tried on mouse testis, which should show a positive result in the interstitial cells and a negative or very weak one elsewhere. Frozen sections of fresh tissue were stuck on to slides coated with albumen by gentle and rapid heating; some were treated with mercuric chloride solution, others passed directly into Schiff's reagent. After 10 minutes the slides were washed carefully in sulphur dioxide water, as prescribed by Verne (1929a, repeated by Lison, 1936b, p. 216), to remove all traces of Schiff's reagent and so prevent the subsequent reformation of basic fuchsin. Sections were also used which had not been subjected to heat.

This technique is unsuitable for all those tissues that will not retain their coherence on thawing after sectioning. The control sections, both heated and unheated, showed a very faint purplish-red coloration throughout, which was also seen in places where there was an appreciable amount of albumen. Schiff's reagent is very difficult to wash out thoroughly, even from sections, and this general coloration is considered to have no significance. The sections treated with mercuric chloride solution showed a much deeper, patchier, and rather bluer coloration, but were too badly smeared to allow its precise location to be determined.

The Feulgen-Verne technique was tried on the same material, but fixation was in saturated mercuric chloride solution, that concentration being considered more suitable, and was for 6 hours only. Treatment with Schiff's reagent gave a positive result which was more feeble than that obtained by the plasmal technique. Gérard's technique, formal-calcium (Baker, 1944) being used as fixative for 6 hours, gave an extremely feeble, almost negative, result with the control sections, and a positive intermediate between the plasmal and Feulgen-Verne results with the sections treated with mercuric chloride.

Adrenals of mature male and immature male and female white rats were used for a more extended trial of Gérard's method. It was found that after

6 hours' fixation, the medulla was almost negative; the cortex showed a positive result only in the zona glomerulosa and the innermost region of the zona reticularis; these results were exceedingly feeble and the intensification with mercuric chloride was very slight indeed. But sections left overnight in water showed a much more positive result in the cortex, which appeared to follow the distribution of lipoids very closely, being most marked in the outer region of the zona fasciculata where the cells are very heavily laden. The picture seen was exactly comparable with those obtained on the same material with Sudan IV or Sudan black (Harrison and Cain, 1947, pl. I, fig. 2). There was no visible difference between the controls and treated sections. The Feulgen-Verne technique (with fixation for 6 hours) gave the same results except that the sections were brighter throughout, and the medulla was as bright as the cortex. Sections left overnight in water showed a considerable intensification of the colouring in the cortex, and a slight reduction in the medulla.

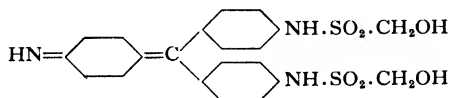
As frozen sections of fresh tissue were of very little use, observations were made macroscopically on small pieces of tissue. Portions of mouse skin, body-wall, liver, kidney, testis, and adrenal were dropped into Schiff's reagent. Colouring was seen only after 20 minutes, and then principally at the cut surfaces. It spread and intensified slowly over a period of hours. But pieces of the same tissues dropped into Schiff's reagent containing mercuric chloride behaved very differently. An intense reaction was seen throughout the tissue (except in the case of liver and of adipose tissue) within 5 minutes, and it had apparently reached its maximum within 15. The colour produced was a very deep violet. This reaction differs remarkably in speed, colour, and intensity from those previously seen.

The relation between this intense reaction and that obtained on fixed tissues was investigated by repeating the experiment with mouse skin, body-wall, kidney, and liver (to act as control), after various periods of fixation in mercuric chloride and in formal-calcium, and comparing the intensity and shade of the resulting coloration. It was found that after 2 hours' fixation in mercuric chloride solution, the reaction was definitely weaker. After formal-calcium, followed by an hour's washing in running water to remove the formaldehyde, only a feeble reddish-purple coloration was produced. After 2 hours in the reagent (Schiff plus mercuric chloride) both lots of tissue were considerably more intensely coloured, though by no means as dark as the unfixed tissue, and the formaldehyde-fixed material was still somewhat redder, though much less so than at first. After 6 hours' fixation, the mercuric chloride-fixed pieces showed a coloration slightly paler than that obtained after 2 hours; liver was slightly more coloured than before. The formaldehyde-fixed pieces were much weaker. After 24 hours, the reaction was not prevented but was comparatively very weak, and there was a strong tendency for the liver to colour.

It would seem, then, that there are two distinct phenomena involved. On the one hand is the intense and rapid reaction given by fresh tissue treated

with a mixture of Schiff's reagent and mercuric chloride, which is progressively reduced by fixation, and apparently affected specially by formaldehyde. On the other is the reaction seen in adrenal cortex, slower, increasing with time, obviously closely connected with the amount of lipid present, and affected comparatively slightly by treatment with mercuric chloride. It appears to be this reaction that is shown by the techniques of Feulgen-Verne, and of Gérard, if fixation is prolonged (more than 3 hours), with a trace of the other if treatment with Schiff is carried out on sections not more than 4 or 5 hours after the tissue is placed in the fixative.

As it is so difficult to wash out Schiff's reagent, and previous fixation is not to be recommended for studying the rapid reaction, an observation on the solubilities of Schiff-compounds (further discussed below) was used in an attempt to improve on the plasmal technique. The compound between formaldehyde and Schiff's reagent is water-soluble and, as will be seen from its formula,



it is a very feeble dye. The following technique was therefore employed:

1. Drop pieces of fresh tissue into a mixture of equal parts of
 - (a) Schiff's reagent diluted with its own volume of sulphur-dioxide water, and
 - (b) a saturated aqueous solution of mercuric chloride.

For controls, drop other pieces into the diluted Schiff's reagent, without addition of mercuric chloride.

Allow the pieces to remain in these solutions for 15 minutes.

2. Remove tissues, wash in sulphur-dioxide water to remove the excess Schiff's reagent without allowing recoloration, and drop immediately into formalin (40 per cent. formaldehyde). Leave for 2 hours.

The formaldehyde-Schiff compound forms rapidly, and the control pieces become almost as dark violet as the others.

3. Wash in running water for 2 hours or more until the control pieces have become only a very pale lilac. This washing also removes the formaldehyde before embedding.
4. Either cut sections without embedding, or embed in gelatine overnight, harden in formalin, cut sections, and mount in Farrants's medium. As the colour begins to pale, these operations should be carried out as quickly as possible.

This method is applicable only to very small pieces of tissue, as penetration by Schiff's reagent is poor. The results obtained were good, the stain being very intense and apparently very selective, especially in skin. It was noticeable that fat droplets were completely negative but the cytoplasm of the fat-cells was heavily positive. Sebaceous glands were negative, the bases of the hair

follicles feebly positive, muscle and myelin sheaths very positive. Remarkably enough, small elements scattered in the connective tissue are feebly positive; these appear to be nuclei of connective tissue cells. The nuclei of the fat-cells also appear to be coloured faintly. A control section should therefore be coloured with Sudan black, to demonstrate the lipoids present, and only positive results obtained on lipoids should be considered. In mouse adrenal, the medulla was negative, the cytoplasm of most of the cortical cells positive, the lipid droplets completely negative. The control sections were colourless in all cases.

Sections prepared by the Feulgen-Verne technique showed a much more feeble coloration or none at all.

B. Experiments with Unsaturated Lipoids in vitro

Oleic and linoleic acids were available for these experiments. In general, it was found that, as might be expected, linoleic acid reacted more rapidly and gave more intense results than did oleic acid. Presumably arachidonic acid would be more reactive still. In interpreting the results it must be remembered that pure lipoids are well known to be much more slowly oxidized than impure ones, and that lipoids in tissues may well react far faster than the same substances when extracted.

Both oleic and linoleic acids will react with Schiff's reagent after about an hour, producing a fine purple, which is certainly not the colour of basic fuchsin. It is worth noting that if a thin layer of oleic acid is allowed to lie on the Schiff's reagent in a test-tube without disturbance, the colour is formed at the interface and shows a strong tendency to diffuse in the aqueous fluid, the rest of the oleic acid being uncoloured. If, however, the tube is continually shaken, the oleic acid gradually becomes coloured, and the Schiff's reagent is only slightly tinged. The same effect can be seen with linoleic acid.

Both oleic and linoleic acids if exposed to the air will become coloured by Schiff's reagent within 15 minutes. After prolonged exposure (several days) coloration takes place within 2 or 3 minutes. Linoleic acid solidifies as it oxidizes, oleic acid does not. Strips of filter-paper soaked in the acids and exposed on clock-glasses protected from dust are convenient for these experiments. If they are exposed to mercuric chloride solution, oxidation is very much more rapid. Linoleic acid exposed to the air for 18 hours colours with Schiff's reagent in 10 minutes. If left in contact with mercuric chloride for that length of time, it colours almost instantaneously. After treatment with saturated mercuric chloride solution for 15 minutes the unoxidized acids showed no shortening of the time taken to colour with Schiff's reagent. Partly oxidized acids showed a very slight shortening and intensification of the colour. The extremely high rate of oxidation of the lecithins is well known, and arachidonic acid is particularly associated with them (Hilditch, 1947). It is possible that a pseudoplasmal reaction might be caused through acceleration of the oxidation of highly unsaturated acids by mercuric chloride.

Unoxidized oleic and linoleic acid can be prevented from oxidizing by treatment with phenylhydrazine, 2-4 dinitrophenylhydrazine, and semicarbazide. The oxidized acids recolour Schiff's reagent in the same time as do samples of oxidized acid untreated with these reagents. As neither the pure nor the oxidized acids will reduce ammoniacal silver nitrate even on boiling, it appears that aldehyde groups are not formed during atmospheric oxidation for at least a week, and that phenylhydrazine and similar reagents, being reducers, can block further oxidation by their presence, as do carotenoids dissolved in fats. It was noticed that the acids became darker and redder in colour, indicating that the phenylhydrazine was being oxidized.

In contrast to the above, palmital and stearyl, after treatment with phenylhydrazine, were completely negative to Schiff's reagent, and remained so after immersion in it for 24 hours. The phenylhydrazine was not decomposed by the acidity of the reagent within this period.

Verne (1929b) remarks that by treatment with phenylhydrazine for 24 hours, the staining is abolished in both the cortex and medulla of the adrenal. This is confirmed for the adrenal of the rat. Staining in the medulla is discussed below.

C. The Status of the Plasmal Reaction as a Histochemical Test

Danielli (1947) has shown that the product of the reaction between Schiff's reagent and desoxyribosenucleic acid is water-soluble, diffusible, and readily taken up by chromosomes when used as a stain, the rest of the preparation being coloured faintly. The colour produced with oleic and linoleic acids is diffusible, as mentioned above, in the Schiff's reagent. Slices of an oleic acid-gelatine emulsion fixed in formaldehyde and carefully washed were coloured and placed in various solutions. The colour appears to be slightly soluble in very dilute acids, more so in stronger ones, but insoluble in alkaline solutions and in alcohol.

The coloured substance produced from Schiff's reagent and acetaldehyde is very slightly soluble in distilled water, insoluble in alkaline solutions, and very slightly in acid ones, but readily soluble in alcohol. It is a very deep violet. The substance produced from Schiff's reagent and formaldehyde is easily soluble in distilled water and dilute acids and alkalis, but only slightly in alcohol. That produced from palmital and stearyl appears to be insoluble in water, dilute acids, and dilute alkalis, but easily soluble in alcohol. In aqueous suspension it shows no dyeing power.

The properties of the formaldehyde-compound were further investigated. Sections of mouse skin placed in an aqueous solution became violet but lost all their colour on washing, except that the hair-shafts remained very faintly purple for some time. Sections of rat adrenal took up the colour on prolonged immersion, the heavily laden fat-cells in the cortex being coloured most deeply. The medulla was scarcely tinged. Immersions up to an hour produced almost no effect. Also, as mentioned above, the colour even when produced *in situ* can be washed out completely.

It appears, then, that while the lower members of this series may be water-soluble in certain circumstances, the higher ones are not, and none have any appreciable tinctorial power. The slightest contact with regenerated basic fuchsin is far more dangerous than an hour's soaking in a solution of one of these substances; fortunately, the colour produced is a very light red, and stains almost all parts of a section indiscriminately. However, as Lison has shown, colour is not always a trustworthy guide. But if one section in a series is coloured much more red (less blue) than others, it should be regarded with suspicion.

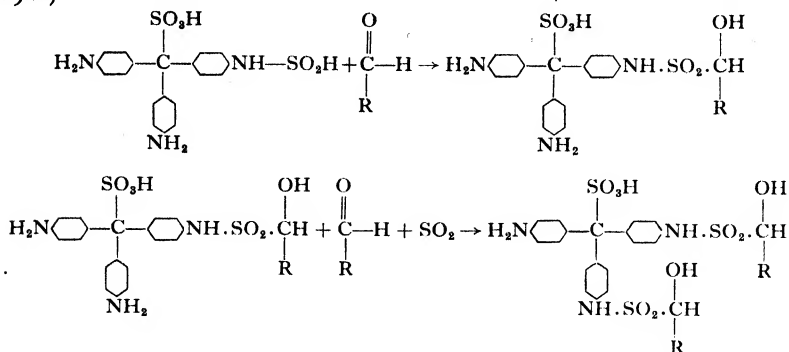
DISCUSSION

It appears that in the past two distinct reactions have been confused under the name of 'plasmal', the intense rapid initial coloration, which is apparently due to 'plasmal' in Feulgen's sense, and the slow reaction, increasing with passage of time and only slightly affected, if at all, by mercuric chloride, which is due to autoxidation products of unsaturated lipoids.

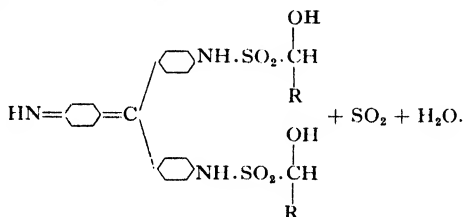
The first, the plasmal reaction, is seen at its most intense when fresh tissue is acted upon by a mixture of Schiff's reagent and mercuric chloride. Schiff's reagent by itself showed no positive result within 15 minutes, or only an exceedingly feeble one, on the tissues examined. This reaction is, as Imhäuser showed, very widespread. Liver is almost negative, but the other tissues examined show intense colorations. Fixation reduces the intensity of the reaction progressively, and it is interesting to note that pieces of tissue give a more intense reaction than do sections cut without embedding from the same pieces before treatment. Palmital and stearal polymerize very readily, and are, in general, very reactive substances. Presumably the increased exposure consequent upon section-cutting assists in their alteration.

Fat globules are totally unaffected by this reaction. In the other, they appear to be the principal sites of it, and stain intensely in the cortex of the adrenal in sections which have been left in water overnight. Verne considers the resulting coloration as a positive result for plasmal. But the distribution of the colour has altered considerably, and the characteristics of the reaction are quite different. It increases in intensity with the passage of time, and mercuric chloride has little or no effect. The work of Gérard and of Lison shows that oxidizers appear in connexion with this reaction in certain cases at least. This is in agreement with part of the chemical evidence on the autoxidation of unsaturated lipoids, during which process many and varied radicals may appear (Markley, 1947; Hilditch, 1947). If it is known that one is dealing with a single substance, or even with two, then possibly they may be identified by the use of a large number of reagents undergoing different types of reaction. In this particular case the number of radicals is unknown, and the reactions available appear to fall into only three groups—reduction, addition, and condensation, as exemplified by ammoniacal silver nitrate, sodium bisulphite, and phenylhydrazine. The reaction of aldehydes with Schiff's reagent is an

addition followed by a condensation, according to Wieland and Scheuing (1920):



which rearranges to:



but as Lison has shown, oleic acid can react, as the elements of water are supplied entirely from the Schiff's reagent. The reaction with silver nitrate, which, as Verne remarks, is not easy to carry out on sections, is also given by ethylene oxides, and addition reactions can also take place across carbon-carbon double bonds; and although phenylhydrazine and similar compounds appear to be specific for the $> \text{C}=\text{O}$ group, their reducing-power can interfere with the reactions of groups other than aldehydes and ketones. Sodium bisulphite and dimedone (Heilbron and Bunbury, 1943) are also reducing agents. In these circumstances no certain conclusion can be drawn as to the composition of reacting mixtures. The only conditions under which any definite statement could be made about reactions in tissues are found when the reaction can be reproduced *in vitro*, and analysis of the extract shows that only one substance is responsible; these conditions have never been satisfied.

In the case of the plasmal reaction, there is one other characteristic, which is of the utmost importance, namely, the speed at which the reaction is produced under the influence of mercuric chloride. It has been shown above that the speed of autooxidation of unsaturated fatty acid radicles can be greatly increased by the presence of mercuric chloride, the normal and the catalysed rates both increasing with the degree of unsaturation. Studies on arachidonic acid are unfortunately not available, and the possibility should be borne in mind that a short treatment with mercuric chloride of fresh tissues containing

it might cause an appreciable intensification with Schiff's reagent. But the speed of breakage of the acetal linkage is so great, as Feulgen showed, that it seems reasonable to treat positive results obtained within 10 minutes on sections or small pieces of tissue as being due to the liberation of plasmal. However, there is no absolute certainty. A prolonged treatment with Schiff's reagent must not be used because the oleic and other unsaturated radicles might begin to react directly.

The evidence that this reaction, produced rapidly under the influence of mercuric chloride, is due to the liberation of palmital, stearal, and other aldehydes from acetalphosphatides, is entirely the work of Feulgen's school, and its interpretation in that sense rests entirely upon this basis. Consequently, in carrying out the reaction care must be taken that the necessary conditions are complied with. Tissues should be handled rapidly to prevent autoxidation, and a control section is essential because, as has already been remarked, a positive result upon fresh tissue untreated with mercuric chloride might be due to any or all of several radicles and the means at our disposal do not allow of a discrimination between them.

This raises an interesting point. Unless the substrate can be shown to be lipid, there is no justification for considering the reaction to be due to plasmalogen and plasmal. A case in point is the medulla of the adrenal. The extremely powerful lipid-colorant Sudan black shows no lipid whatever in this region. Yet it colours, after Verne's technique, as strongly as the innermost region of the zona reticularis and the outermost of the cortex. Prolonged exposure of sections does result in the cortex becoming much more positive, but the medulla becomes more pale. Obviously, this is not a case of the Feulgen-Verne reaction of unsaturated lipids; if it were, the medulla also would become more positive. But equally it cannot be said to be a true plasmal reaction because the medulla is negative with the technique given above. As Verne remarks (1929b), phenylhydrazine abolishes the reaction in both cortex and medulla. But phenylhydrazine is a strong base, and soaking tissues in strong bases reduces considerably their power of being stained by basic dyes. If this were the mechanism, then the nature of the colouring matter is in doubt, because it has the appearance of a product of Schiff's reagent, but, as we have seen, such products do not appear to act appreciably as dyes. Verne claims that it is due to aldehydic substances which diffuse from the cortex into the medulla. The complete proof of this would require that a definite reaction could be produced on extracts of the medulla made with lipid-solvents, and that this could be shown to be the same as that found in the reticularis. A mere suppression of the reaction by treatment with alcohol is not sufficient proof. Alcohol has many effects on tissues besides extracting some of the lipids. There is, however, another possible explanation. Basic precipitates of mercuric chloride solutions will turn deep violet in the presence of Schiff's reagent. Now the colouring of adrenal sections fixed in mercuric chloride is brightest around the free edge of the cortex and the edges of the large fenestrae caused by vigorous fixation in the medulla. It is at least

possible that this distribution is due to unequal conditions of fixation. One may decide that these explanations are unlikely, and it is more reasonable to attribute the reaction to plasmal; but there is no histochemical proof. But where lipoids can be demonstrated in the tissue, and the reaction ceases when they are removed, and mercuric chloride exerts a profound and rapid effect, it is likely that plasmalogen and plasmal are responsible.

The techniques available for demonstrating plasmal histochemically are not completely satisfactory, the demands of the cytologist and chemist being at variance. The reaction can easily be studied macroscopically on small pieces of fresh organs dropped into the reagents. Short fixation is necessary for good results. As mercuric chloride is a very powerful precipitant, the best procedure is probably to fix very small pieces for a short time in a formaldehyde fixative, wash carefully, and carry out the technique on frozen sections in the way usually recommended. But pieces fixed and stained simultaneously according to the procedure given above should also be examined. The reaction in them is far more intense, and far more tissue elements are coloured. This is almost certainly the reason why the Feulgen school, working on fresh sections, found the plasmal reaction so widespread, while Verne, using fixed material, found it far less so. As liver appears to give only the feeblest reaction even when fresh, a piece of it can be carried through with the other tissues under investigation, to act as a general control on the method. It is not denied that a true plasmal reaction can be obtained on pieces fixed for several hours, but it is greatly reduced and may easily be confused with the beginnings of the Feulgen-Verne reaction.

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SUMMARY

1. Two different reactions have been confused under the name 'plasmal'. One, the true plasmal reaction, is due, as its discoverer Feulgen showed, to the liberation of higher aliphatic aldehydes from acetalphosphatides, the reaction taking place very rapidly under the influence of mercuric chloride, the products being coloured deeply with Schiff's reagent. The other reaction is due to the oxidation, by atmospheric oxygen, of double bonds in unsaturated fatty acid radicles. It is affected only slightly, if at all, by mercuric chloride, and increases with exposure of sections or pieces of tissue to atmospheric oxygen.

2. There is no completely satisfactory method for the histochemical demonstration of plasmal. Sections of fresh tissues are not usually suitable for cytological work, and fixation causes a reduction in intensity of the

reaction. Also, the permissible fixatives are not all of the highest quality. Short fixation in a formaldehyde fixative (e.g. formal-calcium) followed by careful washing is probably the best, but the results should be compared with those obtained by the direct reaction of small pieces of fresh tissue. A suitable technique is described.

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The Cell-Theory: a Restatement, History, and Critique

PART II

BY

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PROPOSITION II

Cells have certain definable characters. These characters show that cells (a) are all of essentially the same nature and (b) are units of structure.

THE essence of this proposition can most easily be grasped by considering what would be left of the cell-theory if it were omitted. We should then be in the same position as was Leeuwenhoek (1674), who, having found that a number of tissues consisted of 'globules', was not surprised to find the same structure in milk. This second proposition is concerned with the reasons for supposing that certain objects, called cells, are all to be regarded as strictly comparable with one another and not comparable with globules such as those of milk.

Very gradually, over a period of centuries, it came to be recognized that there is a fundamental living substance, the protoplasm; that this protoplasm commonly occurs in small masses, each provided with a nucleus; and that each of these masses is to some extent separated from its neighbours by a cell-membrane having special characters. Proposition II covers these discoveries and is also concerned with the reasons for supposing that cells are unitary components of organisms and that one cell corresponds with one other cell and not with several. The present paper deals with the discovery of protoplasm and the nucleus. The discussion of Proposition II will be continued in Part III of this series of papers.

The Discovery of Protoplasm

One of the most fundamental facts about cells is that they contain protoplasm as their characteristic constituent, and that, with some partial exceptions that are mentioned on p. 98, this substance never occurs except in

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cells or in objects formed by the transformation of cells. While allowing that the word protoplasm has no absolutely precise meaning, we must acknowledge that there are many substances of which it can be stated with certainty that they are not protoplasm, and that such substances occur commonly between cells, but never constitute cells; while the substance that exists in cells (and in transformed cells) and is called protoplasm has so many positive characters that it is impossible to suppose that we are lumping together under a single name utterly distinct mixtures of organic compounds. This is not the place to give a list of the physical and chemical properties of the substance; we are concerned here only to trace the history of the idea that the cells of plants and animals have a substance called protoplasm as their characteristic component.

The earliest observations and experiments on this substance were not made in connexion with cells. Trembley (1744) made a careful study of the protoplasm of *Hydra* without ever understanding the cellular nature of these animals. He was investigating the microscopical 'grains' (apparently the carotene-granules and nematocysts) that he had discovered. He noticed (p. 56) that when he had teased up a fragment of the body in a drop of water, some of the 'grains' remained bound together by 'une matière glaireuse' (literally, a substance resembling white-of-egg). Trembley stretched a fragment of the body between the points of a quill pen and saw the glairy substance spin out between them (p. 57). He was able to isolate this substance almost completely from the granules. He attributed the cohesion of the granules to the glairy substance. He was also able to stretch a tentacle and to obtain a microscopical view of the part of it lying between two 'grains' (nematocysts): this part consisted wholly of the glairy substance (pp. 63-4). He notes its *transparency* and *tenacity*, the latter being shown by the resistance of the tentacle against breaking when pulled. He attributes to it also the polyp's powers of contraction and expansion.

Duhamel du Monceau (1758, p. 26), in his study of the cellular tissue of plants, mentions the 'substance vésiculaire, ou cellulaire' that fills, as he says, the meshes of the net (i.e. the spaces enclosed by the cell-walls). He remarks that it contracts on desiccation and that it is sometimes coloured.

The discovery of cyclosis by Corti (1774, pp. 127-200) was to play an important part some three-quarters of a century later in leading microscopists to the opinion that the living substance of plants and animals is essentially similar (see p. 95). At the time, the circulation of protoplasm was only an isolated curiosity. Corti uses the name 'Cara' for the various species of freshwater plants on which his observations were made; these included *Chara* and perhaps also *Nitella*. He uses the name *Cara translucens minor*, *flexilis* for the species in which he first saw the circulation of granules in the long internodal cells.

Treviranus (1811, pp. 78-95) first saw cyclosis in 1803. His observations on this subject were made on *Hydrodictyon utriculatum* and *Nitella flexilis* (which he calls *Chara*), among other freshwater plants. It is clear that he

had no knowledge of Corti's discovery. He would appear to have confused cyclosis with the movement of reproductive cells set free by algae, as observed by other authors.

Treviranus calls the protoplasm of freshwater algae 'Gallert oder organische Materie'; he notices the granules in it and mentions that he has seen them also in the cells of the cellular tissue of plants. Indeed, he found each cell of this cellular tissue 'ganz ähnlich' to a segment (Glied) of a conferva (1811, p. 78).

Brisseau-Mirbel (1815, p. 196) uses Grew's word 'cambium' more or less as we might say 'the protoplasm of meristematic tissues'. He describes it as a colourless mucilage that appears wherever new developments are going to occur. He did not understand that it was partitioned into cells, but considered that though a fluid, it contained the 'linéamens' of new structure. In his text-book of histology, Heusinger (1822, p. 41) uses the expression 'Bildungsgewebe (tela formativa)' roughly in the sense of what we should call protoplasm; but his style is reminiscent of Oken's *Naturphilosophie* and he does not give much precise information.

Dujardin (1835) was led to the study of protoplasm by his doubts as to the correctness of Ehrenberg's opinion that the food-vacuoles of ciliates are stomachs joined by an intestine. He was unable to see any tube joining one vacuole to another and his attention was thus directed to the intervening substance. He says that he would perhaps have abandoned these studies, if he had not solved the problem by the discovery of the properties of 'Sarcode'. 'I propose to give this name', he says (p. 367), 'to what other observers have called a living jelly—this glutinous, transparent substance, insoluble in water, contracting into globular masses, attaching itself to dissecting-needles and allowing itself to be drawn out like mucus; lastly, occurring in all the lower animals interposed between the other elements of structure.' It is remarkable that Dujardin at once seized upon most of the important physical characters of the substance he had just named. Indeed, one could hardly improve upon his description in a short statement, except by providing the numerical data that are available to-day. He found (pp. 367-8) that sarcode decomposes gradually in water; unlike albumen, it does not dissolve, but leaves a feeble, irregularly-granular residue. Potash hastens the decomposition; nitric acid and alcohol coagulate the substance and make it white. It spontaneously produces vacuoles within itself. It refracts light much less than oil does.

Dujardin studied sarcode not only in ciliates, but also in *Fasciola* and *Taenia*, in *Nais*, earthworms and other annelids, and in young larvae of insects. He seems generally to have used exudations from rents in tissues for his metazoan material.

Dujardin did not relate his sarcode to cellular structure. Various microscopists, however, began to make short remarks about the substance lying between the nucleus and the boundary of the cell. Valentin (1836), who studied it in nerve-cells, called it the 'Parenchym'. He said that it was 'for the most part a grey-reddish finely granular substance', though transparent

and clear as water in fishes (p. 138). He mentions the 'small, dispersed, separate, round particles' in the cytoplasm of various nerve-cells, and figures them (see especially his Figs. 45 and 49 of Tab. VII). These were almost certainly the vacuoles or spheroids that constitute the basis of the so-called Golgi apparatus, and he should presumably be regarded as the discoverer of this cytoplasmic element.

Schleiden (1838, pp. 143-5) seems to have used the word 'Schleim' in more or less the sense of plant protoplasm; but his attention was so much fixed upon the nucleus and cell-wall, and his ideas on the origin of cells so mistaken, that it is impossible to be sure. Certainly he did not believe his 'Schleim' to be an essential part of the cell, except in so far as the nucleus might be formed of it. He says that it occurs in irregular, granular forms without internal structure, and is stained brownish-yellow or brown by tincture of iodine. He seems to have thought that what we should call the cytoplasm of young cells was a watery fluid *containing* granules of Schleim.

Meyen (1839), like Dujardin, was led to the study of protoplasm by investigating the food-vacuoles of ciliates. Like Dujardin, he denied Ehrenberg's opinion that these animals have stomachs joined by an intestine: there are simply watery vacuoles (Höhlen) in a gelatinous substance. 'The true infusoria', he wrote, 'are bladder-like animals, the cavity of which is filled with a slimy, somewhat gelatinous substance' (p. 75). He mentions (p. 79) that similar vacuoles occur in the 'Schleim' of the cells of plants, particularly in the aquatic filamentous forms, but he is so much interested in the vacuoles that he omits to institute a comparison between the gelatinous substance of infusoria and the Schleim of plants.

Schwann (1839*a*) added little to knowledge of the living substance. He mentions (p. 12) that the cells of the notochord of the frog-larva contain a colourless, homogeneous, transparent substance, which, he says, does not become cloudy at the temperature of boiling water; and he describes the contents of ganglion-cells (p. 182) as being a finely granular, yellowish substance. He gives some account (p. 45) of the 'strukturlose Substanz' of organisms; but this was the supposed Cytoblastem or substance in which cells originate, not the substance of cells themselves. Schwann states specifically (p. 209) that the substance that comes to surround the nucleus in the developing cell is different from the Cytoblastem. He says little about its characters, however, beyond mentioning that it is sometimes homogeneous and sometimes granular.

The first attempt to generalize about the properties of the living substance of plant and animal cells was made by Purkinje on 16 January 1839 at a meeting of the Silesian Society for National Culture. A report on his address was published the following year (Purkinje, 1840*a*). The intrinsic value of his remarks, and the fact that he used the word 'Protoplasma' for the first time in its scientific sense, make it necessary to reproduce a considerable part of what he said. The word Protoplasma had long been used in religious writings in the sense of the 'first created thing'; but it is a surprising—indeed

an astonishing—fact that in introducing the term into science, with a very particular and important meaning, he gives no indication that it was not already in current use in this field. He reserves the word 'Zellen' for cells that have distinct cell-walls, using 'Kügelchen' and 'Körnchen' for those that have not. He uses the word 'Cambium' in the same sense as did Brisseau-Mirbel. He wrote as follows:

'In plant-cells the fluid and solid elements have separated completely in space, the former as the inner, enclosed part, the latter as that which encloses it. In the animal development-centre, on the contrary, both are still present in mutual permeation. The correspondence is most clearly marked in the very earliest stages of development—in the plant in the cambium (in the wider sense), in the animal in the Protoplasma of the embryo. The elementary particles are then jelly-like spheres or granules, which present an intermediate condition between fluidity and solidity. With the advance of development the animal and plant structures now diverge from one another; for the former either tarries longer in the embryonic condition or remains stationary in it throughout life, while in the latter on the contrary the hardening process and the separation of the solid and the fluid progress more rapidly, and come to light first in cell-formation and then in the formation of vessels.'

It will be noticed that although he applies the word Protoplasma only to the substance of the embryonic cells of animals, yet he clearly realizes the correspondence of this substance with that of the adult cells of animals and of the meristematic cells of plants. In the case of the adult plant cell, he regards what we call simply the protoplasm as constituting the fluid part of his Protoplasma, the solid part having separated out as the cell-wall. In another paper, published in the same year, Purkinje (1840b) follows up these ideas by claiming that there should be a 'Körnchentheorie' as opposed to the cell-theory of Schwann, since plants and animals originate from simpler elementary granules, which in plants become changed into cells, while in animals they either remain as they were or change into various forms of fibres. He does not use the word Protoplasma in this paper, but the idea of a substance common to plant and animal cells is implicit in what he writes.

Jones (1841) denied, like Dujardin, that the 'internal sacculi' of ciliates are connected by an intestine (pp. 56-8). He states that the lowest animals consist of a 'gelatinous parenchyma' (p. 6). He speaks of a 'semifluid albuminous matter' loosely connecting the green granules of *Hydra* (p. 21).

Kützing (1841) helped to direct attention towards the protoplasmic part of plant cells, but unfortunately used a confusing terminology. He claimed that each cell of a conferva consists of three elementary parts: the outer 'Gelinzelle', the 'Amylidzelle', and the 'Gonidien'. The first, from his description, was clearly the cell-wall. The second was what von Mohl was later to call the Primordialschlauch, that is the layer of protoplasm lining the cell-wall on the inside. He describes the Amylidzelle as being coloured

brown by iodine; weak acids, alcohol, and drying cause sudden contraction, which cannot be reversed by soaking in water. Kützing made the mistake of supposing that caustic potash converts this layer into starch. The third elementary part was the granular material enclosed by the Amylidzelle (starch-grains, &c.).

A considerable advance was made by Nägeli (1844), who found (pp. 90-1) that there is a slightly granular, colourless 'Schleimschicht' under the whole of the inner surface of the cell-wall of the fully formed cells of green algae and of some fungi. The chloroplasts and starch-grains are attached to it. The whole of the rest of the cells is filled with a water-clear fluid. Nägeli understood that his Schleimschicht corresponded to the Amylidzelle of Kützing, but he objected to the latter's name, firstly because it is not a Zelle (in the sense of 'box'), and secondly on chemical grounds (p. 96). The Schleimschicht, he found, consists of granular slime, which earlier filled the whole cavity of the cell and now lies just within the cell-wall. Its outer surface is smooth, but towards the interior of the cell it forms rather irregular projections. The name 'cell', he insists, is not suitable for such a structure. The Schleimschicht is coagulated by alcohol, weak acids, and water; these are the properties of nitrogenous plant-slime. It is coloured brown by iodine, and it is not changed into starch by potash, as Kützing had said.

These researches of Nägeli to a large extent forestalled the more famous work of von Mohl, who gave the name of Primordialschlauch, or utriculus primordialis, to the protoplasmic layer that lines the inside of the cell-wall of plants (1844, col. 275). This primordial utricle clearly corresponds to the Amylidzelle of Kützing and the Schleimschicht of Nägeli. Von Mohl's term conveys clearly his realization that the cell-wall is not the primary or fundamental part of the cell. He mentions (col. 276) that when a nucleus is present, it lies in the primordial utricle, generally attached to its inner wall; when the nucleus is centrally situated, it is connected to the primordial utricle by slimy threads. The cell-wall stains blue with iodine, while the primordial utricle stains yellowish-brown.

Two years later von Mohl (1846) reintroduced the word 'Protoplasma'. He was quite obviously unaware that Purkinje had already used the word in the same sense. The importance of von Mohl's remarks on this subject justifies rather a long extract. He remarks (col. 73) that if we study a young plant cell, we never find that it contains a watery cell-sap: a viscous, colourless mass, containing fine granules, is dispersed through the cell and is aggregated especially in the vicinity of the nucleus. He thought that this substance was present before the nucleus appeared. 'As has already been remarked,' he writes (col. 75), 'wherever cells are going to be formed this viscous fluid precedes the first solid structures that indicate the future cells. We must further suppose that the development of structure in this substance is the process that initiates the formation of the new cell. For these reasons there may well be justification if, for the designation of this substance, I propose in the word *Protoplasma* a name based on its physiological function.'

(In this translation I have used the expression 'development of structure' to convey the meaning of von Mohl's word 'Organisation'.)

In a footnote von Mohl mentions that Schleiden used the word 'Schleim' in the same sense. Von Mohl objected to this word because it was already used on the one hand loosely for any substance whatever that is of a slimy consistency, and on the other hand in a restricted sense as a synonym for mucus.

He describes (col. 76) how in young cells the nucleus always lies at the centre, surrounded by protoplasm. He proceeds (cols. 77-8) to an account of the origin of the cell-sap. 'Irregularly distributed spaces form in the protoplasm, which fill themselves with watery sap. . . . The older the cell becomes, the more these spaces filled with watery sap increase in size in comparison with the mass of the protoplasm. As a consequence the spaces that have been described flow together into one another.'

It will be allowed that von Mohl had now arrived at a remarkably exact idea of the general plan of a plant cell.

The next necessary step was the discovery that protoplasm is the fundamental constituent of the cells of animals as well as of plants. It might be thought that since the word had first been applied to animal tissues, this step would have been an easy one; but Purkinje's ideas had not received the recognition that was their due, nor had his word 'Protoplasma' been accepted by students of animal cells. The ground gained by Purkinje required to be recaptured.

To Ecker (1848) is due the recovery of the idea that there might be a fundamental substance common to animals of all grades of structure. His object was to discover what there was in lower animals corresponding to the contractile substance of higher animals. He felt that Dujardin's work on sarcode had been disregarded by most histologists. There had been a mistaken tendency to look for parts corresponding to those of the higher animals in the bodies of the lower. 'The body of the Infusoria . . .', he writes (p. 221), 'consists throughout of a simple, homogeneous, half-fluid, jelly-like substance, in which neither cells nor fibres are perceptible—a substance that is sensitive and contractile and in which the essential properties of the animal body are thus not yet confined to particular tissues.'

Ecker concentrated a good deal of his attention on *Hydra*, in which animal he failed to notice the muscular bases of the epithelial cells. He gave the name 'ungeformte contractile Substanz' to the sarcode of Infusoria and the living material of *Hydra*. He found that both were albuminous, soft, either wholly homogeneous and transparent or finely granular; both contained bladder-like spaces or vacuoles; both were in the highest degree elastic and contractile; both insoluble in water, though altered by it; both soluble in potassium hydroxide but hardened and contracted (so he said) by potassium carbonate (pp. 237-8). He claimed to have traced the development of the true striped muscle of the *Chironomus* larva from a completely homogeneous, fibreless, contractile substance.

Ecker's work was important chiefly for its influence on Cohn (1850), who listed the properties of the contractile substance of animals as described by Dujardin and Ecker and then went on to show that this substance was the same as the protoplasm of plant cells. His words (pp. 663-4) are of the utmost importance for the history of the discovery of protoplasm, and must be quoted in full: *'But all these properties are possessed also by protoplasm, that substance of the plant cell which must be regarded as the chief site of almost all vital activity, but especially of all manifestations of movement inside the cell. Not only does the optical, chemical and physical behaviour of this substance correspond with that of sarcode or the contractile substance (which I had the opportunity to study in the Infusoria, Hydra, and Naids)—in particular, both substances are very rich in nitrogen, are browned by iodine and contracted by stronger reagents—but also the capacity to form vacuoles is inherent in plant protoplasm at all times. . . .*

'Hence it follows with all the certainty that can generally be attached to an empirical inference in this province, that the protoplasm of the botanists and the contractile substance and sarcode of the zoologists, if not identical, must then indeed be in a high degree similar formations.

'Accordingly, from the foregoing point of view, the difference between animals and plants must be put in this way, that in the latter the contractile substance, the primordial utricle, is enclosed within a rigid cellulose membrane, which allows it only an internal mobility, normally expressing itself in the phenomena of circulation and rotation—while in the former this is not so.'

Despite this last paragraph, Cohn did not regard the cell-wall as a fundamental part of the plant cell, for he wrote (pp. 655-6): *'In general I comprehend under the expression "primordial cell" that form of the primordial utricle which assumes the aspect of a cell and appears either altogether devoid of a rigid cell-membrane, or independent and isolated from one.'*

It would be difficult to exaggerate the importance of the contribution to our knowledge of protoplasm made by Cohn in the passage just quoted. The contributions made subsequently by Unger, Schultze, Haeckel, and their contemporaries, were amplifications of ideas first formulated by Cohn.

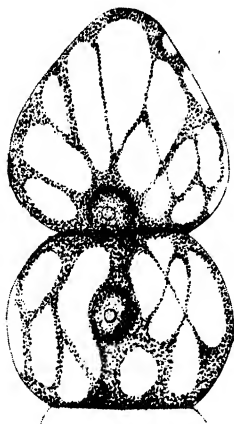
Von Mohl now devoted a book (1851) to the characteristic features of the anatomy and physiology of the plant cell. He remarks (pp. 42, 44) that the protoplasm constitutes a relatively small part of the fully developed plant cell, owing to the large size of the spaces occupied by the cell-sap, which does not mix with the protoplasm. It is difficult to be certain of the exact meaning attached by von Mohl to his word 'Primordialschlauch'. Did he mean the whole of the protoplasmic layer that lies below the wall of the plant cell, externally to the vacuole? or did he mean only the external membrane of this protoplasmic layer? There are remarks on pp. 41-4 which suggest that he was referring to the cell-membrane in the modern sense; but other passages in his writings do not confirm this view, and he does not figure the cell-membrane separately from the protoplasm. He does, however, give a remarkably good figure of typical plant cells, reproduced here as Text-fig. 1.

Remak (1852, p. 53) now adopted von Mohl's botanical word 'Protoplasma' in referring to the substance of the egg-cell and embryonic cells of animals.

The course of progress was now briefly interrupted by an extraordinary episode. T. H. Huxley (1853) made an attempt to discredit not only the view that protoplasm is the fundamental living substance, but also the cell-theory as a whole. He recognized two constituents of tissues: the endoplast (which we should call the protoplasm) and the periplast (intercellular material). His object was to show that life depends primarily upon the intercellular material. 'So far from being the centre of activity of the vital actions', he writes (p. 306), 'it [the endoplast] would appear much rather to be the less important histological element. The periplast, on the other hand, which has hitherto passed under the names of cell-wall, contents, and intercellular substance, is the subject of all the most important metamorphic processes, whether morphological or chemical, in the animal and in the plant.' The endoplast, he says (p. 312), 'has no influence nor importance in histological metamorphosis.' 'We have tried to show', he says (p. 314), 'that they [the cells] are not instruments but indications—that they are no more the producers of the vital phenomena, than the shells scattered in orderly lines along the sea-beach are the instruments by which the gravitative force of the moon acts upon the ocean. Like these, the cells mark only where the vital tides have been, and how they have acted.'

In an important paper Unger (1855) brought strong support to the views that had been formulated five years before by Cohn. After considering the properties of plant protoplasm, and especially its movements, he concludes (p. 282): 'So all this suggests that protoplasm must be regarded not as a fluid, but as a half-fluid contractile substance, which is above all comparable to the sarcode of animals, if indeed it does not coincide in identity with the latter.' Schultze (1858) next described the movement of granules in marine diatoms and compared it with that seen in *Noctiluca* and in the pseudopodia of *Gromia*, Foraminifera, and Radiolaria. In his oft-quoted paper of 1861, which will be considered further in Part III of this series of papers, he mentions that Remak's adoption of von Mohl's word 'Protoplasma' for the substance of animal cells has not been generally copied, and says that he himself will use it henceforth. His example was probably influential.

Schultze's account of the movement of granules in protoplasm was attacked by Reichert (1862*a* and *b*), who claimed that the appearance was illusory. Schultze (1863) had little difficulty in showing that Reichert was mistaken. He proved that the granules of *Gromia* and other rhizopods



TEXT-FIG. 1. Von Mohl's figure of typical plant cells. The figure represents part of a hair (probably a staminal hair) of *Tradescantia Selowii*. (Von Mohl, 1851, Tab. I, Fig. 7.)

are real and that they display characteristic movements during life. He also studied the hairs on the stamens of *Tradescantia* and the parts of other plants in which cyclosis is observed. He found a remarkable agreement. 'The movements in the protoplasm of plant cells', he writes (p. 65), 'resemble those in the pseudopodia of the Polythalamia [Foraminifera] so closely, that when the arrangement of the protoplasm is of the kind that occurs, for example, in the cells of the staminal hairs of *Tradescantia*, no difference between the two kinds of movement is to be discovered.' Schultze also showed that chemical and physical influences had similar effects on plant and animal protoplasm.

The pseudopodia of Foraminifera and Radiolaria lent themselves particularly well to studies of protoplasm. Haeckel (1862) made a careful investigation of its properties as revealed in the latter group. He used his powerful influence in support of Cohn and his successors. He wrote (Häekel, 1868, p. 108): 'The *protoplasm* or *sarcode* theory—the doctrine that the albuminous contents of animal and plant cells (or more correctly, their "cell-substance") and the freely motile sarcode of the rhizopods, myxomycetes, protoplasts, etc., are identical and that in both cases this albuminous substance is the originally active substrate of all the phenomena of life—may well be characterized as one of the greatest and most influential achievements of the newer biology.' After paying tribute to the work of Cohn, Unger, and Schultze, he continues (p. 109): 'I have myself striven for a number of years to support and extend this doctrine by numerous observations.'

Meanwhile a strange figure had entered the field. Beale was independent to the point of perversity; he insisted on using a private terminology of his own; and his writings were marred by their polemical character. Had he understood better how to integrate his own discoveries with those of others, he would have made greater contributions to research on protoplasm. Beale first made his ideas public in a series of lectures to the Royal College of Physicians in 1861 (Beale, n.d.). He distinguished between *germinal* and *formed* matter. The former, to which he ascribed the power of infinite growth, evidently corresponds to protoplasm, while the latter is the inter-cellular material. He regarded an affinity for carmine as a particularly striking character of the germinal matter. Beale's chief interest was in the synthetic function of the germinal matter, and his important contributions to this subject will be discussed under the heading of Proposition IV. The nucleus was for him the quiescent part of the germinal matter. Eventually he accepted the word protoplasm and wrote a book with that name (Beale, 1892); but, wayward to the end, he remarks in it that 'Nowhere in the world is the essential living element a "cell"' (p. 203).

Brücke (1862) brought a new insight into protoplasmic studies. He insisted (p. 386) that protoplasm has 'Organisation'. He denied (pp. 401-2) that it is either solid or fluid. He objected to its being called a slimy or jelly-like substance, for he thought that this was like the description of a medusa as a gelatinous mass by someone who was ignorant of its organization. The

cell-contents must have a complex structure in order to be able to perform the vital activities. His argument was largely deductive and seems to have had little influence on his contemporaries; but it foreshadowed the outlook of a later generation.

Although the Foraminifera and Radiolaria were well adapted for research on the living substance, yet the wholly protoplasmic nature of the Mycetozoa, combined with their fairly large size and ready availability in inland laboratories, made them of predominant importance. In the face of organisms which, in their active, plasmodial phase, contain no 'periplast' whatever, it was impossible any longer to maintain such views as had been put forward by Huxley in 1853. It was this that gave special importance to de Bary's careful study of the group. His description of the protoplasm of Mycetozoa (1864, p. 41) deserves quotation. 'The ground-substance always presents itself as a colourless, translucent, homogeneous mass, exactly similar to the homogeneous contractile substance that is known in the body of amoebae, rhizopods, etc., and is designated as *sarcode*, *unformed contractile substance*, and latterly, like the component of plant-cells which is in many respects analogous, as *protoplasm*. . . . As for its chemical character, rose-red colouring with sugar and sulphuric acid and with Millon's reagent, together with yellow colouring by iodine, indicate a rich content of albuminous substances. Alcohol and nitric acid cause coagulation; in acetic acid the substance becomes thin (blass) and transparent; in potassium hydroxide solution, even when dilute, it dissolves; the same occurs in potassium carbonate solution, though often after the first action of this reagent has produced a contraction.' It will be agreed that this is a remarkably exact short description of protoplasm.

Kühne (1864) brought strong evidence from many sources of the close similarity of plant and animal protoplasm. He studied the living substance in *Amoeba*, *Actinophrys*, *Didymium* (a mycetozoan), in the cells of the connective tissue and cornea of the frog, and in those of the staminal hairs of *Tradescantia*. He observed protoplasmic movements and noted the effects of reagents, of temperature changes, and of the passage of an electric current. He obtained protoplasm from staminal hairs (pp. 100-1) and was so struck by its resemblance to that of *Amoeba* that he called particles of it 'vegetabilische Amöben'.

Huxley's rhetoric was now to be used once more on the subject of protoplasm. Without giving any indication that he had reversed his opinions or had made any observations or experiments that could cause him to do so, he plunged into powerful support of the protoplasm theory. The occasion was a Sunday evening address given in the Hopetoun Rooms, Edinburgh. According to the careful report given in the *Scotsman* (Huxley, 1868, p. 7), he described protoplasm as 'the bases [*sic*] of physical life'; the expression 'the physical basis of life' first appeared in print as the title of his article in the *Fortnightly Review* (Huxley, 1869), which followed closely the Edinburgh address. 'Beast and fowl,' he wrote (pp. 134-5), 'reptile and fish, mollusk,

worm and polype, are all composed of structural units of the same character, namely, masses of protoplasm with a nucleus. . . . What has been said of the animal world is no less true of plants. . . . Protoplasm, single or nucleated, is the formal basis of all life.' The discovery had been made by others: Huxley's contribution was first opposition and then a phrase.

It is to be noticed that the essential similarity between the living matter of plants and animals was discovered by examination of the ground cytoplasm before the existence of mitochondria in both was recognized.

A relatively small point remains. Russow (1884, pp. 578-9) discovered that in the medullary rays of certain plants there exists intercellular material that colours like protoplasm with iodine and dyes. In *Acer* he found thin threads connecting this intercellular with cellular protoplasm. In the same year Fromman (1884) claimed that protoplasm exists in the intercellular spaces of the hypocotyl of *Ricinus*. Like Russow, he said that it reacted to iodine and dyes in the same way as cellular protoplasm. He stated that it often contains single starch grains and small chloroplasts. Intercellular material had already been studied in the cotyledons of the pea by Tangl (1879), who regarded it, however, as secreted matter. The corresponding intercellular substance in the cotyledons of *Lupinus* was seen and figured by Michniewicz (1903), who later saw bridges connecting it with cellular protoplasm (1904). The intercellular material in the cotyledons of *Lupinus* was studied in considerable detail by Kny (1904a). He found that reactions for proteins were positive. He concluded from the bleaching of methylene blue solutions and the blue coloration with guaiacol and hydrogen peroxide that the intercellular material respire. Indeed, he found that it showed the same reactions as cytoplasm in all respects, except that studies with proteolytic enzymes suggested that it contained more protein. His general conclusion was that the substance was in fact intercellular protoplasm. In a second paper (Kny, 1904b) he showed that it is connected with the cells of the cotyledons by narrow bridges. Like Russow, he found that intercellular protoplasm may contain small starch grains.

This subject would be of considerable importance from the point of view of the cell-theory if it could be shown that intercellular protoplasm ever exists in the absence of any connexion with nucleated cells; but this would not appear to be so. The tissues of animals do not provide any close counterpart to the intercellular protoplasm that occurs occasionally in plants.

The Discovery of the Nucleus

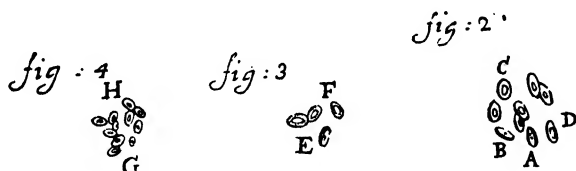
Some of the older botanical writers (e.g. Balfour, 1854) call the nucellus of the ovule the nucleus. This may give rise to misunderstanding. It was stated by Meyen (1839, p. 250), for instance, that both Grew and Malpighi saw the 'Kern' of the 'Eychen' (ovule) of plants. On the same page he uses the word 'Nucleus' as equivalent to 'Kern'. Reference to the relevant parts of Grew's and Malpighi's works shows that there is no question of the

object named being a nucleus in the modern sense (see Grew, 1682, p. 210 and Tab. 82; Malpighi, 1687, p. 71, and Fig. 233 on Tab. xxxvii).

Nuclei were in fact first seen by Leeuwenhoek, whose description of them is contained in a letter sent to the Royal Society in 1700 (see Leeuwenhoek, 1702, p. 556; Leeuwenhoek, 1719, p. 219). The discovery was made in the red blood corpuscles of the salmon. His description of the figure made by his draughtsman (see Text-fig. 2) is as follows:

'Fig. 2 ABCD represents the oval particles of the Blood of a Salmon that weighed above thirty pound.

'AB represents the particles that appeared flat and broad, but did not face the eye directly.



TEXT-FIG. 2. The earliest figure of the nucleus. Red blood corpuscles of the salmon and 'Butt', as represented by Leeuwenhoek's draughtsman. The original numeration of the figures has been retained so that it may correspond with Leeuwenhoek's description as reproduced in the text. (Leeuwenhoek, 1702, Plate opposite p. 220.)

'Those about c came straight upon the eye, and for the most part had a little clear sort of a light in the middle, larger in some than in others, which the Engraver has done his utmost to imitate.'

There can be no doubt that the 'little clear sort of a light' (*lumen* in the Latin version (Leeuwenhoek, 1719)) was the nucleus. Leeuwenhoek also saw nuclei in the blood of a small fish which he calls 'Butt' (1702) or 'Botje'. 'Butt' was a general term in English for flat-fish; in modern Dutch 'bot' is the flounder. Figs. 3 and 4 in Leeuwenhoek's plate (reproduced here as Text-fig. 2) represent the red blood corpuscles of this fish. He refers to the nuclei as 'little shining spots' (1702, p. 557).

Nuclei were seen by some of the early students of Protozoa. Writing to Réaumur in 1744 Trembley gave some sketches of *Stentor* that show the moniliform macronucleus clearly (see Trembley, M., 1943, p. 207). The same observation was made on *Stentor* by Müller (1786, Tab. xxxvi, 8), who calls the macronucleus 'Series punctorum pellucidorum' (p. 262). Müller appears also to have seen the macronucleus in a species of *Colpoda* and a few other ciliates, but there is no indication that he recognized its homology in the different forms. Much later Ehrenberg (1838) saw the macronuclei of many ciliates (*Amphileptus*, *Nassula*, *Chilodonella* ('*Chilodon*'), *Paramacium*, *Spirostomum*, *Stentor*; see his Plates xxiii, xxiv, xxxv, xxxix), but did not understand their nature. It is convenient to mention Ehrenberg in connexion with this early stage of the history, because his work on the subject was not related to the nuclear research of his own period, for in

accordance with his opinion that the ciliates were 'vollkommene Organismen', he regarded their macronuclei as male reproductive glands (see, for example, pp. 262, 332, 352).

Hewson (1777) illustrated the nuclei in the red blood corpuscles of many vertebrates—birds, viper, slow-worm, frog, and fishes—and also saw them in the turtle. He made the understandable mistake of suggesting their presence in his figures of the red blood corpuscles of mammals. He was the first to see the nucleus in the blood corpuscle of an invertebrate. 'If one of the legs of a lobster be cut off,' he writes (p. 40), 'and a little of the blood be caught upon a flat glass, and instantly applied to the microscope, it is seen to contain flat vesicles, that are circular like those of the common fish, and have each of them a lesser particle in their centre, as those of other animals.'

Fontana (1781) would appear to have been the first to have seen nuclei in tissue-cells other than those of blood. He made a microscopical study of the slippery substance that coats the skin of eels and described the 'globules' or 'vesicules' contained in it, which were almost certainly epithelial cells derived from the epidermis. 'One saw', he says (p. 276), 'a little body internally, situated in different parts of each globule.' He means that there is not a characteristic position for the little body within the cell. His figures (e.g. Fig. 9 of Plate 1 in Vol. 2) strongly suggest that the 'petit corps' was the nucleus. Indeed, Fig. 10 shows what seems to be a nucleolus within the nucleus. Fontana says, 'The vesicle *a* in Fig. 10 represents one of the vesicles of Fig. 9, in which one observes an oviform body [the nucleus], having a spot (tache) in its interior.' This 'tache' is probably the earliest illustration of a nucleolus. A life-like view was obtained of these cells because they lay in a medium of suitable osmotic pressure. The early observers were accustomed to tease up tissues in water, in preparation for microscopical examination; and it was only when nature provided a suitable medium and thus made the addition of water unnecessary that good views of tissue-cells were obtained.

When Purkinje discovered the germinal vesicle of eggs, he had no means of knowing that there was any correspondence with the 'lumen' seen in the blood corpuscles of fishes or with the 'petit corps' in the epidermal cells of eels. The discovery was not widely known until five years after it had been made. The Faculty of Medicine at Breslau had decided to congratulate Blumenbach in 1825 on the fifteenth anniversary of his taking his doctor's degree, and they wished to send him an original scientific paper to mark the occasion. Purkinje's offer to write a memoir for this purpose was accepted. It was made available to the world at large in 1830 (see Purkinje, 1830 and 1871). The germinal vesicle of the hen's egg was described in this memoir as follows (1830, p. 3):

'Thus the scar [germinal disk] of the ovarian egg contains a special part, peculiar to itself, a vesicle of the shape of a somewhat compressed sphere. This vesicle is limited by a very delicate membrane and filled with a special

fluid, perhaps connected with procreation (for which reason I might call it the germinal vesicle); it is sunk into a white breast-shaped projection composed of globules and perforated in the middle.' This remarkable passage contains the earliest mention of the objects later to be named the nuclear membrane and nuclear sap.

Coste (1833, col. 243) showed that the egg of the rabbit contains a vesicle corresponding to that discovered in the hen's egg by Purkinje. He later published a monograph (Coste, n.d.) giving figures of the nucleus of the rabbit's egg. In the description of his Fig. 2 he labels the nucleus 'vesicle analogous to that which Purkinje has demonstrated in birds'. Bernhardt (1834), who knew of Coste's work, found the nucleus, or 'vesicula prolifera' as he called it, in the egg of ruminants and of the rabbit, squirrel, bitch, cat, mole, and bat. He gives figures showing the nucleus in the eggs of several of these, and remarks that with certain precautions even a 'tiro' could see it. He says (p. 27) that it is round or nearly round or oval and has a sharp outline. The contents are fluid.

Meanwhile, nuclei had been discovered in plant cells. Bauer made drawings in 1802 which showed them in the cells of the loose tissue lining the canal of the stigma of the orchid, *Bletia Tankervilleae*. These drawings were unfortunately not published until much later (Bauer, 1830-8; see Tab. VI). Nuclei were probably seen from time to time in plant tissues without anyone guessing that they had any general significance. For instance, Meyen (1830, Plate III) shows what look like nuclei in the pith of the stem of *Ephedra*, though he himself regarded them as consisting of resin-like material. Brown, who knew of Bauer's drawing of *Bletia*, was the first to recognize that the nucleus is of more than sporadic occurrence, and it was he who coined the name by which this part of the cell has been known ever since. His words are as follows (Brown, 1833, pp. 710-11): 'In each cell of the epidermis of a great part of this family [Orchidaceae], especially of those with membranaceous leaves, a single circular areola, generally somewhat more opaque than the membrane of the cell, is observable. . . . This areola, or nucleus of the cell as perhaps it might be termed, is not confined to the epidermis, being also found . . . in many cases in the parenchyma or internal cells of the tissue. . . . I may here remark, that I am acquainted with one case of apparent exception to the nucleus being solitary in each utriculus or cell.' Brown also saw the nucleus in various cells of Liliaceae, Iridaceae, and Commelinaceae, and in a few cases also in the epidermis of dicotyledons.

The nucleolus, which had been recorded by no observer since Fontana, was now discovered by Wagner (1835) in the oocytes of various animals (*Ovis* (Fig. 2 on Tab. VIII), *Salmo*, *Phalangium*, *Anodonta*, *Unio*). He called it the Keimfleck or macula germinativa. The recognition of the nucleolus was important, because it helped in the identification of nuclei.

From 1836 onwards reports came repeatedly of the existence of nuclei in animal cells. Purkinje (1836) announced that the Körnchen (cells) covering the choroid plexus (apparently of man) are each provided with a small

'Körperchen'. Valentin (1836a, p. 97) introduced the word nucleus into the literature of animal cytology. Writing of the cells of the epithelium covering the vessels of the choroid plexus of the brain, he says: 'But each of them contains in the middle of its interior a dark, round kernel (Kern), a structure that reminds one of the nucleus that occurs in the plant kingdom in the cells of the epidermis, of the pistil and so forth.' Valentin classifies the epithelia, distinguishing those in which the cells are nucleated from those in which (as he supposes) they are not (p. 96). In a passage of quite extraordinary interest he makes a careful comparison between the egg and the nerve-cell. The latter he calls the formative sphere (Bildungskugel). 'But in what an astonishing way', he exclaims (pp. 196-7), 'does the basic idea of the form of the unfertilized egg correspond with the basic idea of the structure of the formative spheres!' He compares the membrane of these cells with the vitelline membrane, their 'Parenchym' (cytoplasm) with the early yolk, and their nucleus with the germinal vesicle; and he says that a 'Keimfleck' (nucleolus) occurs in both. In a second paper published in the same year (Valentin, 1836b), he again uses the word nucleus, stating (p. 143) that every cell without exception in the epithelium of the conjunctiva of man contains one. He mentions also that the nucleus itself here contains 'a perfectly spherical particle'.

The year 1837 saw the publication of a book of the first importance by Henle (1837), who now described nucleated cells in very diverse human tissues, including even the skin of the glans penis. He uses the word 'Cylindri' when referring to cells of columnar epithelium, but elsewhere uses 'Cellulae'. In describing his Fig. 4 he refers to the 'Cellulae nucleatae' of the human conjunctiva. He illustrates the nuclei of the epithelium of the trachea particularly clearly (Fig. 10). He mentions (p. 4) that the nucleus sometimes contains granules. It is not too much to say that this work, with that of Valentin (1836a), marks the beginning of an epoch in cytology, the epoch of the *nucleated cell*. Purkinje's name for the nucleus of the egg was, however, not readily relinquished. Siebold (1837) noticed the nuclei in the eggs and blastomeres of nematodes, but called them the 'Keimbläschen' and the 'Purkinjesche Bläschen' in the former case (p. 209) and 'hellen Flecke' in the latter (p. 212). He calls the nucleolus of the egg the 'Keimfleck'. These names, with their counterparts in other languages, persisted long afterwards, even when the homologies of the objects named were well understood.

The year 1838 brings us face to face with Schleiden and Schwann. To assess their significance in the advancement of cytology is a difficult task for the historian, and a task that has often been lightly undertaken. Too much credit has undoubtedly been given to them by some, and a reaction against exaggerated praise has produced a literature of rather superficial belittlement. It is necessary to realize in what field their chief contributions lay. They lay exactly here, in the part of this second proposition that is concerned with the nucleus. If there had been no Schleiden and no Schwann there would have been considerable delay in the general realization by

biologists that the possession of a single nucleus is a characteristic feature of most of the cells of animals and plants. Their work, taken together, provided most powerful evidence that there is a correspondence or homology ('Uebereinstimmung', Schwann called it) between the cells of the two kinds of organisms. Their ideas were far from being so original as has often been supposed; for Schleiden had his precursor in Brown, and Schwann in Purkinje, Valentin, and Henle. The three latter had made great advances in animal cytology during a period in which Brown's work on plant cells was scarcely being followed up, and Schleiden's contribution, therefore, represented a more sudden advance than Schwann's; and Schleiden was also ahead of Schwann and communicated his ideas to him in conversation. Indeed, one of Schleiden's most important functions was to act as a stimulus to Schwann: for one can scarcely read the writings of the two men without realizing that Schwann had the greater mind and made much the more massive contribution. These facts stand out even if we deliberately ignore the polemical character of much of Schleiden's writings. It must be allowed that Schleiden had too much influence on Schwann, for the latter took over, without sufficient investigation, his erroneous views as to the origin of cells. That, however, is beside the point for the present: we are here concerned with the great influence of these two men in getting the nucleated cell recognized as the fundamental building-stone of most organisms.

Schwann tells us (1839a, p. 8) that during the course of his work on the nerves of the tadpole of the frog he saw the cells and nuclei (Kerne) of the notochord. In his report on the subject (Schwann, 1837) he said nothing about the notochord; but the image of the notochordal cells remained in his mind. 'One day, when I was dining with Mr. Schleiden,' he tells us (Schwann, 1884, p. 25), 'this illustrious botanist pointed out to me the important role that the nucleus plays in the development of plant cells. I at once recalled having seen a similar organ in the cells of the notochord, and in the same instant I grasped the extreme importance that my discovery would have if I succeeded in showing that this nucleus plays the same role in the cells of the notochord as does the nucleus of plants in the development of plant cells.' The two scientists repaired at once to the anatomical institute in Berlin in which Schwann worked. Here they examined together the nuclei of the notochord, and Schleiden recognized the close resemblance to the nuclei of the cells of plants.

Neither had yet published. Schwann was the first to do so, but it will be convenient to begin with Schleiden's contribution, the 'Beiträge zur Phyto-genesis' (1838). It is difficult to escape from a sense of disappointment on reading Schleiden's paper. There is nothing about a 'cell-theory' in it; it is solely concerned with plants; and it contains a great deal of error in connexion with the origin of cells, together with much that is of secondary interest. In one respect, however, it was of first-rate importance: the regular occurrence of nuclei in the young cells of phanerogams was here for the first time demonstrated. Schleiden thus focused attention on the nucleus as a

characteristic component of the cell. He was also the first to discover the nucleolus of plant cells, without realizing that it corresponded with the 'Keimfleck' already known in both germinal and somatic cells of animals. He calls it a small body (Körper, p. 141), but does not name it. He regards it as 'consistenter' than the rest of the nucleus. He shows it in several figures (see especially Fig. 25 on Tab. III). Beyond all this, Schleiden produced some ideas on cellular individuality, which will be considered under Proposition V.

Schwann's great contribution was his massive array of evidence that there is an 'Übereinstimmung' between the cells of plants and animals. He himself concentrated upon the latter, relying on the researches of Schleiden for his knowledge of plant cells. We have already seen in the discussion of Proposition I that others had previously suggested such a correspondence between the plant and animal cell; but Schwann was struck with enormous force by the fact that each contains a corresponding object, the nucleus, itself containing a corresponding organelle, the nucleolus, which he called the 'Kernkörperchen'. It seems clear that he reached his conclusions from his own studies of animal cells and from discussion with Schleiden, before he knew of the discovery by Purkinje, Valentin, and Henle that the nucleated cell is a common constituent of animal tissues. He went much farther than these three: he found the nucleated cell to be not simply a common constituent, but the fundamental basis of structure. He founded his 'Zellentheorie' (1839a, p. 197) chiefly on his own discoveries. His contributions to the problem of cellular individuality will be mentioned under Proposition V.

In his first cytological paper (1838a) Schwann notes the strong resemblance to the cellular tissues of plants shown by notochordal tissue and cartilage, which he had studied in the larvae of the spade-footed 'toad', *Pelobates fuscus*. He mentions the nuclei of both kinds of cells, each containing one or more 'Kernkörperchen'. He regards the embryo as cellular: 'Since therefore the serous and mucous layers of the blastoderm consist of cells and the blood corpuscles are cells, the foundation of all organs that appear later is composed of cells.' He mentions ganglion and pigment-cells and describes the cellular nature of the lens of the eye and of cancerous growths. This paper was published in January 1838. In it Schwann refers to the forthcoming article by Schleiden (the 'Beiträge'), and claims that the latter's statements about the way in which plant cells multiply are applicable also to animals.

Schwann begins his next paper (1838b) by stressing the importance of the nucleus in showing the correspondence between animal and plant cells. Most of the observations reported here were made on pig embryos. He records the cellular nature of horny matter, of the lining of the amnion and allantois, the surface of the chorion, the alveoli for the teeth, and the surface of tooth-pulp: all consist of cells with nuclei. He is puzzled, naturally enough, by striated muscle. He finds nuclei in the cells of the kidney, salivary and

lacrimal glands, liver, and pancreas. He notes the nucleated cells in connective tissue and considers the white fibres as projections from them. He records the cellular nature of feathers. 'So the whole animal body, like that of plants,' he remarks, 'is thus composed of cells and does not differ fundamentally in its structure from plant tissue.' In his third contribution on this subject, published in April of the same year (1838c), he deals with the cellular structure of cartilage, and shows that nail, fat, and unstriated muscle consist of or develop from nucleated cells. The paper ends with an appendix by J. Müller, in which the existence of nucleated cells in pathological growths (osteosarcoma, &c.) is recorded. The valid factual part of Schwann's great book, which was published in the following year (1839a), consists largely of a reiteration of what he had already made known in these three papers. It contains, however, a considerable amount of interesting theoretical matter, which will be discussed at the appropriate places in future parts of this series of papers.

Meanwhile, Purkinje, Henle, and Valentin had continued to make discoveries in the same field. Purkinje (1838a) mentions the 'Centralkern' in the 'Körnchen' (cells) of the liver. He also gives (1838b) an excellent figure of nerve-cells from the black substance of the cerebral peduncle of man. This is reproduced here (Text-fig. 3) so as to give a visual impression of what others were doing in the year in which Schleiden and Schwann made their results known. The nucleus, nucleolus, and pigment are well seen in the figure.

Henle followed up his book (1837) with a paper (1838) in which he gave a detailed description of the cellular nature of the epithelia of the human body, including even the lining of the blood-vessels. He is, of course, familiar with the nucleus and the nucleolus; the former he here calls the 'Kern' and the latter, unfortunately, the 'Nucleus'. A useful reminder of the state of knowledge at the time of Schleiden's and Schwann's contributions is provided by the fact that Henle's paper occurs before Schleiden's 'Beiträge' in the same volume of the journal. About the same time Valentin (1838) contributed a curious paper on the differentiation of the cells of the human embryo into their definitive forms. He tried to follow the behaviour of the nuclei (Zellenkerne) during differentiation. What he writes on this subject contains much error, but he was striking out on an important new line and at least he made it clear that the nucleus is far less liable to modification during differentiation than the rest of the cell. Next year Valentin made an unequivocal statement of the correspondence of the nucleated cells of plants



TEXT-FIG. 3. Purkinje's figure of nerve-cells, published in the same year as Schleiden's and Schwann's first publications on cells. The cells are from the black substance of the cerebral peduncle of man. (Purkinje, 1838b, Fig. 16 on Plate opposite p. 174.)

and animals (1839a, p. 133). He remarks that Schwann has completed the comparison between them.

Valentin now introduced the word 'Nucleolus', in the course of an abstract of Schwann's book (Valentin, 1839b, p. 277). He does not mention that he is coining a word, but simply says, 'In mammals the cartilage-corpules appear to constitute the whole cell and as such to contain nucleus and nucleolus.' (Turner (1890a, p. 11) is wrong in saying that Schwann introduced the word.)

The story of the nucleus may be rounded off by mention of Nägeli's demonstration (1844) that this organelle is a characteristic component of the cells not of phanerogams only, but of all kinds of plants from algae upwards. Thenceforth there was seldom any difficulty in recognizing nuclei; appeal was made especially to the nucleolus as a distinguishing feature in cases of doubt. The use of stains in microtechnique was repeatedly rediscovered during the years 1848-58, as I have told elsewhere (Baker, 1943), and this naturally gave a great impetus to the study of what is usually the most stainable object in cells. Huxley's attempt (1853) to discredit the nucleus is therefore all the more extraordinary. He tells us (p. 297) that Schleiden's belief in the existence of nuclei in all young tissues is 'most certainly incorrect'. The nucleus, he says (p. 298), 'has precisely the same composition as the primordial utricle.' Little attention, however, appears to have been paid to him. From the forties onwards the position of the nucleus in cytology was secure: it was regarded as an essential constituent of the cell. It is to be noted that this conclusion was reached long before there was any general agreement that protoplasm was also a necessary constituent. This may at first seem strange; but it must be recollected that the nucleus is obviously easier to recognize than protoplasm, on account of its having morphological as well as physical and chemical characters.

The discussion of Proposition II will be continued in Part III of this series of papers. I thank Prof. A. C. Hardy for his valuable criticism of the typescript of this paper, and Miss O. Wilkinson for conscientious clerical assistance.

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On the Functional Morphology of the Alimentary Tract of Some Fish in Relation to Differences in their Feeding Habits: Anatomy and Histology

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With Three Plates

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INTRODUCTION

THE present work is the continuation of a series of studies (Al-Hussaini, 1945-7) attempting to correlate the structure of the alimentary canal with the feeding habits of teleosts. These earlier studies were based on distantly related species and thus the differences observed and described might well be due to congenital factors rather than to purely adaptive causes—of this no certain answer can yet be given. The present study is an attempt to exclude congenital factors as nearly as possible by choosing three closely allied species

with different feeding habits, namely, the mirror (king or spiegel) carp (*Cyprinus carpio* L.), the roach (*Rutilus rutilus* (L.)), and the gudgeon (*Gobio gobio* (L.)), species which will hereafter be referred to by their generic names only. From the point of view of the general problem this series suffers from a slight defect in that all three are capable of dealing with a mixed diet, that is, they are strictly speaking omnivorous. Nevertheless, the mirror carp (similar in feeding habits to the common carp) ingests more plant than animal food (Couch, 1865; Day, 1880-4; Regan, 1911), in the gudgeon animal food preponderates in the diet (Yarrell, 1841; Day, 1880-4; Susta, 1888; Regan, 1911, Hartley, 1940, 1947), while the roach is omnivorous in the true sense (Grevé, 1897; Regan, 1911; Hartley, 1940, 1947), and they have been customarily described as herbivorous, carnivorous, and omnivorous feeders according to the material which composes the greater bulk of their diet. An attempt was made to justify these conclusions experimentally, but unfortunately, owing to unavoidable circumstances, the number of fish which could eventually be spared was very small, so that not more than some three or four individuals could be fed on any one specialized diet. Such results as were obtained, however, supported the view above expressed. It may be noted further that the carp and the gudgeon both seek their food from the bottom.

The present account deals simply with the comparative anatomy and histology of the mouth, pharynx, and gut of the three types. This will be followed by an account of the cytological, histochemical, and physiological aspects of the problem so that the 'functional morphology' and the adaptive correlations may thereby acquire a firmer basis.

HISTORICAL SURVEY

Good historical reviews of early work have been written by Oppel (1896), Sullivan (1907), and Biedermann (1911), and it is not proposed to add much to them beyond what is necessary to provide an appropriate background for the present study. Amongst the more important works that have appeared since these reviews may be mentioned Jacobshagen (1911, 1913, 1915, 1937), who extended Eggeling's (1907) earlier study of the detailed configuration of the intestinal mucosa, from both taxonomic and adaptive points of view, while Pictet (1909) working on similar lines studied, in particular, the gut mucosa of five Cyprinid species. Dawes (1929) described the histology of the gut of the plaice and defined the pharynx, oesophagus, and rectum, pointing out certain changes in both cytoplasm and nucleus of the columnar epithelial cells which he associated with secretory activity: Of the more recent workers directly concerned with Cyprinids, mention must be made of Rogick (1931) working on *Campostoma anomalum*, and of Curry (1939) who studied the common carp—*Cyprinus carpio communis*. Sarbahi (1940) differentiated a caecal from a pyloric portion of the intestinal bulb and described conical cells which hang freely into the lumen of the rectum in *Labeo rohita*. McVay and Kaan (1940) investigated the goldfish—*Carassius auratus*—and discerned the changes which occur in the cells of the intestinal epithelium, already

referred to by Dawes, but offered no explanation of the phenomenon. Finally, Klust (1940) described the changes which occur as development proceeds in several Cyprinid species. Authors who have attempted to correlate the structure of the alimentary tract with the feeding habits of the fish have already been reviewed in a previous paper (cf. Al-Hussaini, 1945).

MATERIAL AND METHODS

This work was carried out in the Department of Zoology, Sheffield University, the roach and gudgeon being supplied by the Sheffield Corporation Water Works from their ponds surrounding the city, while the mirror carp came from the Surrey Trout Farm (Haslemere). The Red Sea types used for comparison were collected personally from the neighbourhood of the Marine Biological Station at Ghardaqa, while other supplementary types came from various English waters.

The techniques employed are numerous, and it will be more convenient to refer to them in the appropriate sections of the text with which they are individually concerned. When an anaesthetic was needed for experimental work urethane was used, while the fishes were normally killed by a blow on the head.

In the following account the omnivorous roach is described as the central type and then compared with the other two species.

THE ANATOMY OF THE ALIMENTARY TRACT

The Protractile Apparatus of the Mouth

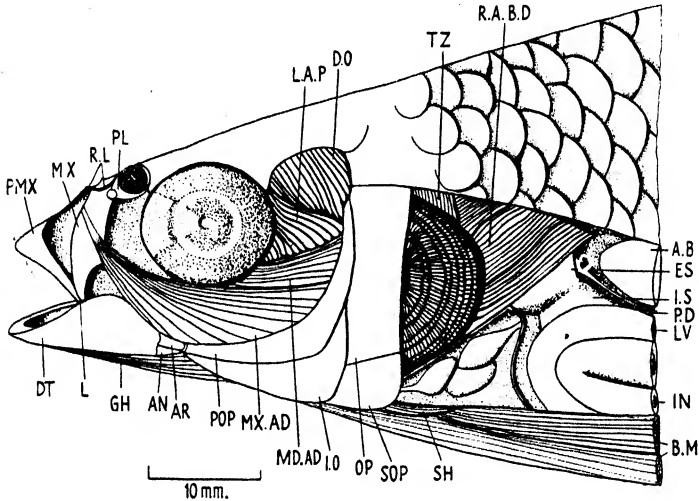
The mouth of *Rutilus* (Text-fig. 1) is small and agrees with the 'normal' type of mouth described by Gregory (1933) in *Micropterus*, except that in the *Rutilus* both jaws lack teeth. Mouths of this type have a moderate gape and moderate protrusibility. The maxillae are excluded from the gape and the premaxillae develop articular and ascending processes.

This latter process, or 'spine', is attached to the ethmoid by the rostral ligament (R.L.) containing a small cartilaginous nodule. According to Eaton (1935), this nodule ensures that the ligament shall bend in precisely the same way each time the jaw retracts. The protrusibility of the mouth depends largely on the length of the ligament which both allows, and checks, the forward thrusting of the upper jaw, and also on the length of the premaxillary spine; the larger the spine the more protrusible the mouth (Eaton, 1943).

The ventral end of the maxilla (MX.) is attached to the premaxilla by a short flexible ligament near the angle of the mouth. Dorsally the two maxillae meet and form a groove in which the premaxillary spine and ligament slide back and forth. The maxillae are also loosely attached to the vomer and palatine, so that when premaxillae are thrust forwards the maxillae do not impede but rather assist this process, since this dorsal maxillary hinge allows their ventral ends to thrust forwards with the premaxillae.

In the lower jaw the dentaries are held together at the symphysis by a very short ligament allowing a slight amount of movement.

The advantage of a protrusible mouth to *Rutilus* is that it increases the range over which the fish can take food as well as enabling it to engulf small prey or other food particles entire by a quick snap, and thus compensates for the absence of teeth on both jaws and palate.



TEXT-FIG. 1. Lateral view of head of *R. rutilus* showing protraction of the mouth. The lacrymal, the posterior part of the operculum with its branchiostegal rays, and part of the pectoral girdle have been removed, and the anterior part of the body cavity exposed.

In *Gobio* the mouth is larger than in *Rutilus*, and a barb is attached to each jaw angle. The upper jaw is larger than the lower so that the mouth opening is directed distinctly downwards. Both the premaxillary spine and the rostral ligament are longer and hence the mouth is more protrusible than in *Rutilus*. When the jaws are fully protruded the mouth opening is completely ventral, a feature obviously connected with the bottom-feeding habit of the fish. When roach and gudgeon were kept together the roach would immediately dash at any food (e.g. insects) thrown in, but the gudgeon ignored it completely until some had sunk to the bottom of the tank. The differences in the shape of the mouth and in the length of its constituent parts represent, therefore, the morphological expression of differences of feeding habits.

In the herbivorous *Cyprinus* the head is relatively larger in proportion to the rest of the body than in either of the other two species. The length of the rostral ligament and premaxillary spine, and hence the protrusibility of the mouth, is intermediate between *Rutilus* and *Gobio*. There are four barbs,

shorter than those of *Gobio*, around the mouth, one from each jaw angle and a dorsal pair from the skin covering the maxillae.

Although *Cyprinus* feeds more easily from the bottom, yet it can rise to take food from the surface and hence combines the feeding habits of *Rutilus* and *Gobio*. These differences in feeding habits are also reflected in other structures. Thus Evans (1940) showed that in *Rutilus* the eyes and optic lobes are much larger than in either *Gobio* or *Cyprinus*, but in the latter two species the facial and vagal lobes (concerned with taste) are much better developed than in *Rutilus*. Thus *Rutilus*, with its superior vision, is able to snap its food freely in the water while *Gobio* and *Cyprinus* normally seek it from the bottom and have developed barbs richly supplied with taste-buds for this purpose (cf. p. 126).

The Pharyngeal Dental Apparatus

The fifth branchial arch is reduced to a single strong bone on each side, the 'os pharyngeus inferior' (Goodrich, 1930, p. 440), usually referred to in cyprinids as the 'pharyngeal jaw'. The modified arch in *Rutilus* (Pl. II, fig. 7a, b, c) is typical of the type described as 'omnivorous' by Chu (1935) in Chinese cyprinids. Each half of the complete arch is about three times as long as it is broad, its posterior limb is about one and a half times as long as the anterior, while at the angle between them there is a distinct lateral projection just behind the second tooth. The posterior surface is pitted (number of pits variable) and wing-like. An edentulous process curves strongly dorsally, making an obtuse posterior angle. This process is compressed and sometimes tapering. It is directed medially, reaching the otic region of the skull where it meets its fellow from the opposite side; thus the two processes form an arch bounding the opening to the oesophagus.

In *Gobio* (Pl. II, fig. 7f) the length of each pharyngeal bone is about four and a half times the width, the posterior limb is not longer than the anterior limb, while the pitted surface is narrower than in *Rutilus*, dimensions which agree with those given by Chu (1935) for the carnivorous species of the sub-family Gobioninae in Chinese waters.

In *Cyprinus* the pharyngeal bones are relatively well developed (Pl. II, fig. 7d, e). The length of each is only about two and a half times the width and the two limbs are of about equal length. They are thus massive bones compared with the other two types.

In all these types, the teeth are affixed to the medio-ventral aspect of each pharyngeal bone. In *Rutilus* they are close-set and uniserial, usually five in each row. Each has a cutting edge. The two posterior teeth (4 and 5) have recurved tips, forming hook-like processes directed backwards. These teeth, together with the third, are strongly compressed, but the first two teeth are stouter and more or less conical with pointed tips. Sometimes an additional tooth develops on the left side, a fact already noted by Hubbs and Hubbs (1944). In their discussion of 'bilateral asymmetry' in vertebrates they quote the roach (*R. rutilus*) as an example. Out of 104 specimens examined 79 had

asymmetrical teeth, the extra tooth being always on the left, never on the right. It should be noted that the extra tooth is only found in well-grown specimens, never in small ones. It therefore appears to be added at a later age.

The teeth in *Gobio* are biserial, each bone has a medial row with four or five teeth and a lateral row with two only. Each tooth is wedge-shaped and hooked as in the posterior teeth of *Rutilus*. The medial teeth are stronger than the lateral and, with the exception of the first which is small, of approximately equal size. The teeth of the lateral row lie opposite the third and fourth teeth of the medial row.

In *Cyprinus* the teeth are arranged triserially (2:1:1). In the mirror carp the rows are not quite so sharply defined as in the common carp in which sub-species there is also an extra tooth (3:1:1). The free ends of the teeth are rounded, somewhat pointed, but never hooked. All four teeth converge and collectively form a very strong, stud-like prominence, a feature regarded by Chu (1935) as of a highly specialized herbivorous type.

Small teeth may be found embedded in the mucous membrane of the pharynx in the vicinity of the pharyngeal bones. Chu regards these as accessory growing teeth destined to replace old teeth if these are lost.

A horny pad is developed in the dorsal wall of the pharynx below the occipital region of the skull opposite to the pharyngeal teeth. In *Rutilus* this is more or less ovoid in plan (Pl. I, fig. 1, H.P.) and double convex in transverse section (Pl. III, fig. 8) and fits loosely into a corresponding fossa in a special masticating process of the basioccipital. Its free surface is rugose and the pharyngeal teeth bite against it. This pad, together with the alternating disposition of the teeth, which are thus enabled to shear the food, completes an exceedingly efficient masticating apparatus.

The pad of *Cyprinus* is similar to that of *Rutilus* but even harder, but in *Gobio* the pad, trapezoidal in plan, is not nearly so well developed and is much softer (it can be easily pierced with a needle).

It follows from the above comparison that the entire pharyngeal dental apparatus is best developed in the herbivorous type (*Cyprinus*), facilitating the comminution of plant material, in the carnivorous *Gobio* the hooked teeth help to secure and tear the prey, while finally the omnivorous roach is strictly intermediate with both hooked and shearing teeth.

The gill-rakers are short in *Rutilus* and *Gobio*, but are longer in *Cyprinus*. Long gill-rakers characterize the majority of bottom-feeders which stir up the mud—a habit which the carp is known to have (Kyle, 1926). Thus, for example, they are well developed in the bottom-feeding *Mugil auratus* and *Upeneus barberinus* (Al-Hussaini, 1947b) and also in *Mulloidides*, which is a bottom-feeder and shovels sand, and in *Scarus*, a coral feeder which grinds its food into a pasty mass (Al-Hussaini, 1946, 1945). The shortness of the gill-rakers in *Gobio* is therefore surprising, especially when one reads in Regan (1911), describing how *Gobio* secures its food, that it 'gropes and grubs' for it.

The Mechanism of Deglutition

The jaws are opened to admit food mainly by the action of two pairs of muscles, namely, the *geniohyoidei* (Text-fig. 1, GH.), a pair of stout muscles arising from the first and second branchiostegal rays and ceratohyals and passing forwards in close apposition to each other to be inserted on the inner surface of the dentary, and the *sternohyoidei* (Text-fig. 1, SH.) which arise somewhat laterally from the cleithra and pass forwards and towards the middle line to be inserted on the dorsal surface of the urohyal on either side of its dorsal crest. Thus by drawing the hyoid ventrally and caudally the sternohyoidei augment the action of the geniohyoidei.

The entry of prey or food into the mouth may be still further assisted by the action of the *levator arcus palatini* (Text-fig. 1, L.A.P.) which arises from the otic region of the skull and is inserted into the hyomandibular in *Gobio* and *Cyprinus* and into the hyomandibular and pterygoid in *Rutilus*. Thus by raising the hyomandibular (and pterygoid) the mouth cavity is enlarged and the food sucked into the mouth.

The closing of the mouth after the entry of the food is effected by the *adductor mandibulae*, a large, complex muscle lying immediately below the skin in the cheek region. In *Rutilus* two portions are distinguishable, the *maxillaris* (Text-fig. 1, MX.AD.) and the *mandibularis* (MD.AD.). The first of these arises from the preoperculum, passes below the eye, and is inserted by a strong tendon into the lateral surface of the dorsal portion of the maxilla. Its contraction lowers the maxilla slightly and thus holds the mouth firmly closed against the pull of the second portion, the *mandibularis*, on the lower jaw. The *mandibularis* portion arises from the hyomandibular, preoperculum, and quadrate, and runs immediately below the eye to its insertion by means of a strong tendon on the inner surface of the mandible. In *Gobio* both portions are further subdivided, the *maxillaris* into external and internal moieties, and the *mandibularis* into three more or less distinct parts. *Cyprinus* is intermediate in that the *mandibularis* portion is simple like that of *Rutilus* while the *maxillaris* is double and resembles that of *Gobio*.

In the living fish it may be observed that the roach is able to snap its mouth closed more quickly than either the gudgeon or the carp, an observation that may be correlated on the one hand with the single insertion for each portion of the adductor muscle in *Rutilus* against the multiple insertions in the other two species, and on the other with the feeding habits of the fish. Whereas *Rutilus* secures food which is moving freely through the water, *Gobio* and *Cyprinus* feed in a more leisurely manner by probing for sedentary food particles in the mud.

Once the food is securely within the mouth it may be assisted on its passage to the pharyngeal teeth by the narrowing of the mouth cavity. This is accomplished by two pairs of muscles. First, the *hyohyoideus* lies below the 'tongue' in the region of the geniohyoideus. Its action is to raise the 'tongue' and with it the floor of the mouth. In *Gobio* and *Cyprinus* this muscle comprises two distinct portions, with a greater extension ventral to the gullet

than in *Rutilus*. The second muscle constricting the buccal cavity is the *adductor arcus palatini*. It lies deep to the *levator arcus palatini* and arises from the parasphenoid and pro-otic and is inserted into the hyomandibula, pterygoid, and palatine, thus it draws these elements inwards and forwards. The area of insertion of this muscle is broader in *Gobio* and *Cyprinus* than in *Rutilus*.

As the food passes backwards into the pharynx the pharyngeal jaws are opened to admit it by the action of the *pharyngo-clavicularis*, a complex muscle with an 'externus' and an 'internus' portion. Both portions arise from the anterior surface of the cleithrum and pass in a generally dorso-medial direction. The 'externus' portion is inserted into the posterior surface of the anterior limb of the fifth branchial arch while the 'internus' portion is inserted partly on the ventral surface of the anterior extremity of the fourth branchial arch and partly in a similar position on the fifth arch. The action of this muscle is augmented by that of the trapezius (Text-fig. 1, TZ.) a strong muscle arising from the occipital and otic regions of the cranium and inserted into the tip of the posterior limb of the pharyngeal jaw. It is best developed in *Cyprinus*. The muscle, antagonistic to these two, which draws the teeth together and on to the surface of the horny pad is the *retractor arcus branchialis dorsalis* (Text-fig. 1, R.A.B.D.), a very powerful muscle, triangular in outline, arising from the masticatory process of the basi-occipital just lateral to the horny pad (cf. Pl. III, fig. 9), and inserted on the caudal surface of the posterior limb of the pharyngeal bone. Again it is best developed in *Cyprinus*, less so in *Rutilus*, and much less in *Gobio*.

Two other muscles are worthy of mention in connexion with feeding, namely, the *constrictor pharyngeus*, which passes laterally and somewhat caudally from the mid-ventral line of the pharyngeal wall, and the *intermandibularis* (Pl. I, fig. 2, IM.). This is a very small muscle (some 0.64 mm. wide in a roach 7 cm. long) passing between the medial surfaces of the dentaries; it is partially covered by the geniohyoidei. In the Cyprinids, where only small food particles are taken, this muscle remains small and only a slight movement is possible between the rami of the lower jaw (cf. also Takahasi, 1925), but in predatory types of mouth, e.g. *Pterois volitans*, the two dentaries may be widely separated during swallowing (Al-Hussaini, 1947b) when large prey is taken. Thus in this fish the intermandibularis is large, as it is in Catostomids (Takahasi, 1925; Edwards, 1926) and Siluroids (Takahasi, 1925).

Apart from the muscles described above, those operating the operculum and gill arches during respiratory movements by varying the hydrostatic pressure within the mouth and pharynx are bound to affect the passage of the food within.

As already mentioned small pieces of food are normally taken by a quick snap (less marked in *Gobio*), but where the prey is larger (e.g. earthworms) the fish first suck the food with great eagerness and then thrust it out again. The process is repeated several times till finally the fish, as it evades others, retires into a corner and indulges in what appears to be mastication. The

respiratory movements are accelerated and in particular the opercula are swung farther out than in normal respiration. The earthworm may be recovered from the gullet a short time later torn into pieces.

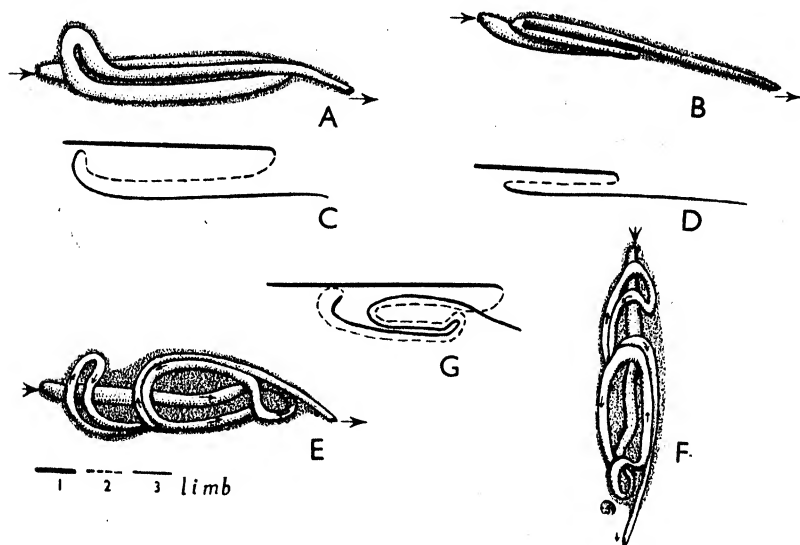
Thus in summary, the cycle of events occurring just before and during the capture of prey is as follows. The mandible is depressed and the premaxillae accordingly thrust forwards by the contraction of the genohyoideus and sternohyoideus, the motion of the mandible being transferred to the premaxillae by virtue of the ligamentous joint between them (Text-fig. 1, L.) the motion being limited by the length of the rostral ligament. As soon as the prey is seized and contained in the buccal cavity the two adductor mandibulae are simultaneously contracted, thus closing the mouth, the mouth cavity is narrowed by the contraction of the adductor arcus palatini and by the elevation of the mouth floor, thus pushing the food back towards the pharynx. The mouth is then opened again and the buccal cavity and the pharynx are dilated (the latter by the various muscles attached to the gill arches), and water is sucked in through the mouth. The prey is thus washed still farther backwards. In the next movement, that of closing the mouth, the pharyngeal floor is raised by the contraction of the constrictor pharyngeus, thus forcing the prey into the region of the teeth. Here, through the action of the retractor arcus branchialis dorsalis, trapezius, and pharyngo-clavicularis, the teeth can work alternately against each other, squeezing the prey between the two 'jaws' and against the horny pad, and finally pushing it backwards into the oesophagus. This 'masticating' action seems to be the more laborious part of the feeding process as observed in the living animal. It should be noted that a Cyprinid fish can hold its food and break it down by its teeth concurrently with the respiratory movements; in other words, deglutition and respiration do not interfere with each other. This is made possible by the position of the 'pharyngeal jaws' at the entrance to the gullet, that is posterior to the gills.

The Intestinal Tube

In all three examples the oesophagus, more or less cylindrical in form, occupies an oblique position in a cranio-caudal direction (Text-fig. 1; Pl. I, fig. 1, ES.), owing to the postero-ventral growth of the masticating process of the basi-occipital. It is remarkably short (4 mm. in a roach 175 mm. in length) and is delineated from the intestine by a constriction. The pneumatic duct (Text-fig. 1, P.D.), carrying a small dilatation at its extremity, opens into the dorsal aspect of the oesophagus.

The alimentary canal posterior to the oesophagus increases suddenly in diameter forming an intestinal swelling (Text-fig. 1, I.S.) as in all other Cyprinids so far described by various authors and in *Atherina* (Al-Hussaini, 1947a) and several labroid fishes (Al-Hussaini, 1947b). The swelling is a straight tube, gradually decreasing in diameter as it extends caudally, close to the dorsal body-wall, nearly as far as the posterior end of the air-bladder, from which point it curves sharply forwards again making one siphonal loop.

In *Rutilus* the distal limb of this loop, which reaches as far forwards as the transverse septum, is of smaller but constant diameter. It turns caudally once more and, coursing on the left of the siphonal loop, terminates at the anus. Sometimes the bend where the second and third limbs join one another has an upward tilt (Text-fig. 2A). The last portion of the third limb is tapering and corresponds to the rectum. Now it is true that in the cyprinids there is no ileorectal valve separating the intestine from the rectum as in most



TEXT-FIG. 2. The intestine as it appears *in situ* from the left side; A, *R. rutilus*; B, *G. gobio*; E, *Cyprinus carpio*; F, the intestine of *Cyprinus carpio* from the ventral aspect, C, D, and G diagrammatic representations of figs. A, B, and E respectively.

teleosts, but the fact that the last, tapering portion of the intestine has different histological features (cf. p. 134) is considered sufficient justification for designating it rectum. For a contrary view, reference should be made to Jacobshagen (1937), who considers that cyprinids, mormyrids, and some others do not have the mid-gut differentiated from the hind-gut.

In *Gobio* (Text-fig. 2B, D) the distal limb of the siphonal loop is short, the intestinal swelling extending only as far as the middle of the anterior lobe of the air-bladder before turning back. In *Cyprinus* (Text-fig. 2E, F, G) the intestine is longer and its looping more complex. The intestinal swelling, or first limb of the intestine, extends caudally throughout the whole length of the body-cavity, the intestine then curves sinistrally upwards and forwards to the middle of the body-cavity, and then backwards again in a sigmoid manner, becoming dextral once more as it approaches the posterior extremity of the body cavity. Here it doubles forwards again and courses anteriorly to the

septum transversum, taking an upward and sinistral course, then back once more with a hairpin loop, then forwards to the middle of the body-cavity and finally, with another sharp loop, it turns caudally and ultimately opens to the exterior at the anus. In the morphological sense the intestine of *Cyprinus* may still be regarded as formed of one and a half siphonal loops, as in the other two species, but the second and third limbs are strongly curved in the middle and shifted to the right (Text-fig. 2, compare G with C). This description was made from specimens of about 12 cm. in length, but according to Smallwood and Smallwood (1931) the intestine becomes much more coiled in large specimens of the common carp. It should be noted that the coils of the second and third limbs lie to the left of the first limb in all cases and that the proximal limb bears the swelling and is nearly straight in all three species, while the second and third limbs are shortest in the carnivorous *Gobio* and longest in the herbivorous *Cyprinus*.

The liver (hepatopancreas) grows round most of the intestine. The gall-bladder lies between the anterior lobe of the swim-bladder and the intestinal swelling, and its duct opens into the right side of the latter very slightly posterior to the oesophagus. The pancreatic duct accompanies the bile-duct, but it is difficult to find in gross anatomy.

Fatty tissue fills up the spaces between the three limbs of the intestine along its entire length and contains microscopic pancreatic alveoli embedded within it.

The blood-supply to the intestine is derived from a coeliaco-mesenteric artery which leaves the aorta just posterior to the pharyngeal dental apparatus.

The Mucosal Folds

In all three forms the mucous membrane along the inner edge of the upper jaw is produced into a crescent-shaped maxillary valve (Pl. I, fig. 1, MX.v.), papillated on its ventral surface. Contrary to the majority of teleosts an opposing mandibular valve is wanting, and thus the maxillary valve must operate against the floor of the mouth.

On the roof of the mouth there are several longitudinal folds which may be branched, or even swollen, and in the region opposite the 'tongue' they are papillated. In *Gobio* there are two distinct protuberances a little posterior to the maxillary valve. These will be referred to as 'palatine cushions'.

In all three species the pharynx is differentiated into two distinct regions, an anterior (Pl. I, fig. 1, A.PH.) and a posterior (P.PH.) proportioned to each other as 2:1. Functionally, the anterior pharynx containing the gill-slits is concerned with respiration, while the posterior pharynx containing the horny pad and pharyngeal teeth has an alimentary purpose. The mucosal folds increase in complexity as they run backwards (cf. Pl. I, fig. 1).

In *Cyprinus* the roof of the anterior pharynx is very 'fleshy' and exhibits an interesting response to mechanical stimulus—found to a less extent in the other two species. When pierced with a needle it swells considerably and then

gradually subsides. The reaction is shown even by a recently killed specimen from which histological samples were being taken.

The mucosal folds lining the intestine vary somewhat from one region to another. Various observations have been made concerning their pattern in sundry fishes by earlier workers, notably by Eggeling (1907) and Klust (1940).

In *Rutilus* there are some 10–12 longitudinal folds in the oesophagus which are continuous with the folds of the intestinal swelling. In the intestinal swelling the folds run in various directions, branch, and reunite. They are relatively broad, being about 0.33 mm. across when seen in surface view (Pl. I, fig. 1). The folds are particularly dense and crowded in the first centimetre of the swelling but become less so farther back. By the time the proximal part of the third limb of the intestine is reached, the mucosal folds are predominantly transverse in direction although they may still decussate. The distal five-sixths of the third limb exhibits a transverse zigzag pattern in the mucosal folds reminiscent of the arrangement of myomeres in the dogfish. The folds are also much thinner when seen in section (about four folds to the millimetre) and the mucosa has a brownish colour which is highly characteristic of this part of the intestine. About a centimetre before the anus is reached a faint line may be seen from the mucosal surface passing transversely round the intestine at the place where, in most fishes, the ileorectal valve occurs. The mucosal folds of the part of the intestine between this line and the anus (i.e. the rectum) assume a longitudinal direction, show only a few interconnexions, and become thicker once more.

In *Gobio* the mucosal folds resemble those of *Rutilus* except that those of the third limb of the intestine assume a longitudinal rather than zigzag orientation, and in the rectum the interconnexions between them become more numerous. In *Cyprinus* the folds of the third intestinal limb resemble those of *Gobio* but the condition in the rectum is more like *Rutilus*.

The four segments of the intestine can therefore be conveniently recognized by the following characteristics: the intestinal swelling, or first limb, has a greater diameter and the mucosal folds are complex, the second limb has a smaller and uniform diameter and less complex mucosal folds, while the third limb, similar in diameter to the second limb, is characterized by its brownish colour and narrow mucosal folds, and finally the rectum is tapering and its mucosal folds are longitudinal.

The Surface Area of the Intestinal Mucosa

The surface area of the intestinal mucosa is of great importance since it is this area which is concerned with the absorption of food. Warren (1939) adopted an excellent technique for measuring this in the dog which was modified by Wood (1944) and applied to cats and rats. The basic principle of the method is to inflate the intestine to a known and constant pressure (60 cm. of water for the dog) with saline and then fix it in this condition and finally cut micro-sections of it in transverse and longitudinal planes. These can be projected at a known magnification on to paper and the linear dimen-

sions of the serosal surface and of the mucosal surface in both directions can be measured by a rotometer. If now SC = serosal circumference, SL = serosal length, SA = serosal area, MC = mucosal circumference, ML = mucosal length, MA = mucosal area, then in general $\left(\frac{MC}{SC} \times \frac{ML}{SL}\right) > \frac{MA}{SA}$ for an irregular surface. However, as Warren shows, a serviceable approximation for the mucosal area (MA) can be obtained by the following formula, where EA = the estimated mucosal area. $\frac{EA}{SA} = \frac{MC}{SC} + \frac{ML}{SL} - 1$. ML, MC, SC, and SL are all measurable quantities, while SA is easy to calculate if the length of intestine under review is assumed to be a cylinder. This last assumption is not quite true for the fish gut since at one end the intestinal swelling and at the other the rectum are of inconstant diameter, and further, since it has been shown that the entire gut is absorptive (*vide infra*) the mere introduction and ligation of cannulae into the ends will produce an unavoidable error into the measurements of serosal and mucosal length. Nevertheless these errors are more or less constant for all three types examined and the data have therefore at least a comparable significance between them. Using Wood's modification, the precise procedure was as follows. The intestine was cut into two parts, viz. the first limb (swelling) in one part and the remainder of the intestine in the other. One end of each piece was attached to an end of the horizontal limb of a \perp -shaped glass tube, the vertical limb of which was of such a length as to give the desired hydrostatic pressure to distend the intestine. The free ends of the two pieces of gut were then joined to the two limbs of a Λ -shaped glass tube, the upright limb of which was connected to a wash-bottle to which a sphygmomanometer bulb was attached. On gently raising the pressure in the wash-bottle by means of the bulb the fluid contents of the bottle could be driven at a steady and constant pressure through the two portions of intestine simultaneously and up the vertical glass tube till it trickled out of the open end of this tube. The contents were first washed from the gut in this manner using the isotonic solution recommended by Young (1932) for fresh-water fishes followed by 10 per cent. formalin as a fixative. The data thus obtained are given below in Table 1.

Several interesting relationships may be deduced from this table. First the relative length of the gut (R.L.G., column 3) is nearly constant for each individual species, a feature already noted in other fish, but in *Rutilus* it is smaller than is usual for omnivorous species (1.3-4.3) while *Cyprinus* also has a smaller R.L.G. than the normal range for herbivorous fish (3.7-6.0); *Gobio*, on the other hand, lies within the normal range for carnivores (0.5-2.4) (Al-Hussaini, 1947b). The small R.L.G. of *Rutilus* and *Cyprinus* together with the simplicity of the gut (absence of stomach and pyloric caeca) may be peculiar to these species, but a considerably more extensive survey would be needed to settle this point.

The ratio between the estimated mucosal area and the serosal length (columns 6 and 9) is greater for the intestinal swelling than for the rest of the

TABLE I. Measurements of the Absolute Length of the Intestine, Mucosal Area, &c., in *Rutilus rutilus* (R1-6), *Gobio gobio* (G7-12), and *Cyprinus carpio* (C13-15)

Fish no.	1	2	3	4	5	6	7	8	9	10	11	12
	Length of fish less caudal fin in mm. = FL	Total length of intestine in mm. = SL	SL/FL = R.L.G. (relative length of gut)	Length of 1st limb of intestine in mm. = SL ₁	Estimated mucosal area of first limb in cm. ² = EA ₁	EA ₁ /SL ₁	Length of 2nd and 3rd limbs of intestine in mm. = SL ₂	Estimated mucosal area of 2nd and 3rd limbs of intestine in cm. ² = EA ₂	EA ₂ /SL ₂	Total estimated mucosal area of intestine in cm. ² = EA ₁ + EA ₂	Weight of fish in gm. = WF	EA/WF = mucosal coefficient = QM
R1	95	100	1.05	20	8.36	4.18	80	23.85	3.18	32.21	14.0	2.39
R2	142	155	1.09	35	25.27	7.22	120	60.48	5.04	85.65	56.0	1.57
R3	155	165	1.06	45	42.08	9.35	120	74.16	6.18	116.24	75.5	1.53
R4	158	140	0.90	35	27.48	7.85	105	71.19	6.78	98.67	66.0	1.50
R5	185	183	1.00	48	54.91	11.44	135	102.51	7.63	157.42	104.0	1.51
R6	185	185	1.00	46	51.38	11.17	139	114.85	8.27	166.23	105.5	1.58
G7	101	69	0.68	21	11.13	5.30	48	15.55	3.24	26.68	11.0	2.42
G8	103	74	0.72	25	13.75	5.50	49	17.98	3.67	31.78	13.1	2.41
G9	104	75	0.72	25	15.73	6.29	50	18.75	3.75	34.48	16.0	2.15
G10	116	88	0.76	30	22.53	7.51	58	18.44	3.18	40.97	19.2	2.13
G11	119	101	0.85	32	21.54	6.73	69	25.46	3.69	47.00	22.0	2.14
G12	120	102	0.85	32	20.54	6.42	70	28.91	4.13	49.45	23.2	2.13
C13	75	140	1.87	20	7.66	3.83	120	21.48	1.79	29.14	11.5	2.53
C14	76	140	1.84	20	9.62	4.81	120	24.96	2.08	34.58	13.5	2.56
C15	98	180	1.84	30	16.92	5.64	150	33.00	2.20	49.92	20.2	2.50

intestine, a feature obviously to be correlated with the complexity of the mucosal folds. Further, although the estimated mucosal area is *absolutely* greater in the larger compared with the smaller fish (cf. columns 1, 10, and 11) because the mucosal foldings grow with the fish, nevertheless it is *relatively* smaller (cf. R1 with R5 and R6). In this connexion it must be remembered that the animal in its growing stage needs a relatively greater quantity of food and hence has a greater absorptive area per unit of body-weight. This ratio between the mucosal (absorptive) area and the body-weight (mass of tissue requiring food), which I have called the *mucosal coefficient*, is expressed numerically in column 12. So far as mammals are concerned Wood (1944), working on cats and dogs of practically equal weight, found by measurement that the ratio is constant in both mammals, while Cori (1925), using growing rats of varying weight, deduced a constant ratio from the quantities of sugar absorbed.

Referring again to the Cyprinid fish, it may be seen that in *Gobio* the mucosal coefficient becomes nearly constant by the time the fish has attained a length of 110 mm. and a weight of about 15 gm. Again, if the mucosal coefficients of the three fish are to be compared and related to their varied diets it should be done with fish of approximately the same weight. This has been done in Table 2.

TABLE 2. *Relative Length of Gut and Mucosal Coefficient of Three Species of Fish of approximately Equal Weights*

Species	No. in Table 1	Intestinal length in mm. = SL	Body-weight in gm.	Estimated mucosal area in cm. ² = EA	Mucosal coefficient	R.L.G.
<i>Gobio gobio</i> . . .	G8	74	13.1	31.78	2.41	0.72
<i>Rutilus rutilus</i> . . .	R1	100	14.0	33.52	2.39	1.05
<i>Cyprinus carpio</i> . . .	C14	140	13.5	34.58	2.56	1.84

An examination of the table shows that *Cyprinus* has the highest R.L.G. and *Gobio* the lowest, and although the mucosal area follows the same sequence the difference is very much less marked. Thus one is led to the important deduction that the shortness of the intestine may be compensated by an increase in the complexity of the mucosal foldings. It should, nevertheless, be borne in mind that the longer intestine, although having a mucosal coefficient very little greater than the short one, probably has the advantage of retaining the food for a longer period and may still be regarded as an adaptation to herbivorous diet provided that the comparison is made between closely related species living under fairly similar conditions.

THE HISTOLOGY OF THE ALIMENTARY TRACT

For this study serial sections were made from specimens measuring 65-100 mm. long. The material was fixed and decalcified simultaneously in

Boling's modification of Bouin's picro-formol (Cowdry, 1943, p. 188) and stained in Delafield's haematoxylin-eosin, certain details being subsequently verified by using Mallory's triple connective-tissue stain, Mayer's mucicarmine, or Giemsa's method for paraffin sections. Individual cell types were studied by the maceration method of Goodrich (1942). In addition to these methods the oesophagus and intestine were removed from specimens of different sizes and fixed in Bouin, Maximow, or Susa and stained with Heidenhain's iron-alum haematoxylin-eosin.

The Buccal Cavity

The buccal cavity of *Rutilus* is lined by a stratified epithelium containing taste-buds and mucus-secreting cells and resting on a basement membrane supported by a stratum compactum (Pl. I, fig. 2). The sub-epithelial connective tissue is differentiated into a tunica propria in juxtaposition to the epithelium and a deeper, looser tissue known simply as the submucosa, a condition similar to that found in all fishes studied earlier (Al-Hussaini, 1945-7).

At the entrance to the mouth cavity the epithelium is thick and the outermost layers are formed of strongly cornified polyhedral cells. In these respects it resembles the epithelium of the outer skin but differs from it in that both mucus-secreting cells and taste-buds are absent; both of these structures occur in the skin. Further, the 'Kolbenzellen', characteristic of the skin of certain teleost families (Uhlich, 1937), are absent. Such cells have been described from the buccal epithelium of *Calamoichthys* (Purser, 1926). They have a central nucleus embedded in a clear cytoplasmic fluid which does not show the staining reactions characteristic of mucus.

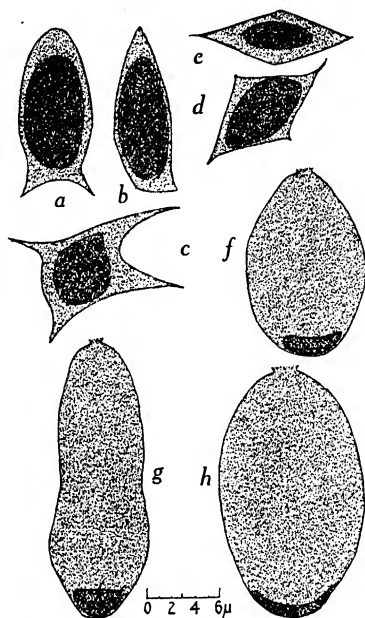
A very short distance within the mouth opening taste-buds begin to appear in the sections and rapidly become numerous as one passes backwards; they are particularly numerous on the ventral surface of the maxillary valve.

According to Herrick's (1903-4) view of the direct correlation between the density of taste-buds and the delicacy of the sense of taste in fishes, *Rutilus* should have a well-developed sense of taste in both the skin and the mouth. In *Rutilus* each taste-bud is a flask-shaped structure measuring about 100μ by 20μ and in general structure similar to those already described in *Mulloides* (Al-Hussaini, 1946).

By the time the middle of the maxillary valve is reached taste-buds have become the predominant feature of the buccal epithelium and are found all over the mouth cavity except on the dorsal surface of the maxillary valve. On the other hand, mucus-secreting cells, which have already made their appearance at this level, are plentiful just where taste-buds are absent or sparse, namely, on the dorsal surface of the maxillary valve and the roof of the mouth. Towards the posterior end of the valve taste-buds and mucus-secreting cells are nearly equally distributed on both the roof and floor of the mouth. At the sides of the mouth, which in transverse sections appear as two recesses, both elements are scarce.

The mucus-secreting cells are mostly of the pyriform type (cf. Al-Hussaini, 1945, p. 368, for terminology of mucus-secreting cells) and are concentrated along the crypts of the buccal epithelium rather than on the folds. Although assuming no particular pattern they become more numerous as one passes caudally towards the pharynx while the taste-buds show a reverse tendency (Text-fig. 4).

A possible explanation of this differential distribution is that the mucus secreted by numerous cells on the opposing surfaces of the dorsal aspect of



TEXT-FIG. 3. Constituent cells of the stratified epithelium of the bucco-pharynx of *R. rutilus* obtained by Goodrich's maceration method: (a) from the stratum germinativum; (b) from the second layer; (c, d) from intermediate layers; (e) from the superficial layer; (f) a pyriform and (g, h) sacculus mucus-secreting cells.

the maxillary valve and the roof of the mouth prevents their adherence and facilitates the movement of the valve during respiration. They are otherwise absent from the extreme anterior part of the mouth since lubrication of the food is not necessary until after the food is in the mouth, and swallowing has commenced. The taste-buds, on the other hand, are concentrated very early so that the food may be immediately sampled.

The mouth floor, or 'tongue' region, shows no special histological features which differentiate it from other areas of the mouth.

The cells in the basal layer (Text-fig. 3a) are formative and from them the other cell types are derived. The cells of the intermediate layers are frequently polyhedral and have their angles drawn out into short processes which, by

interlocking with similar processes from neighbouring cells, presumably give a firmer and tougher structure to the entire epithelium. Within the layer of cells immediately adjoining the basal layer certain spheroidal cells may be found, each with a short process housing the nucleus. Similar cells may be found in the succeeding layers as the surface is approached, but they tend to get larger and the nucleus becomes compressed at the base rather than contained in a process (*f, g, h*). It would thus appear that the pro-mucus-secreting cells are differentiated along with the stratified cells from the basal layer, and that mucus secretion is commenced very early in the course of their differentiation, the amount of secretion being gradually increased as the cells approach the surface.

The basement membrane, stratum compactum, tunica propria, and sub-mucosa are similar in general structure to those described in the earlier papers. In the floor of the mouth, in the region of the 'tongue', the submucosa contains large adipose cells which persist back to the heart region.

In both *Gobio* and *Cyprinus* the barbs on the lips are very richly supplied with taste-buds, but, in addition to these, taste-buds are much more abundant both on the lips (Pl. I, fig. 3) and in the buccal epithelium than in the roach.

The order of the three fish on the basis of the abundance of taste-buds is *Cyprinus*, *Gobio*, *Rutilus*. It is surprising to read in Curry's account (1939) that taste-buds are not numerous in the floor of the mouth of the common carp and that they 'are more numerous on the flap than in any part of the buccal cavity'. By 'flap' she presumably means the maxillary valve. In contrast to this the mirror carp possesses very numerous taste-buds over the entire lining of the buccal cavity *except* on the dorsal surface of the maxillary valve (Pl. I, fig. 2). In fact I have not yet examined a fish with more abundant taste-buds than the mirror carp, and, further, they are of larger size than those of either *Gobio* or *Rutilus*.

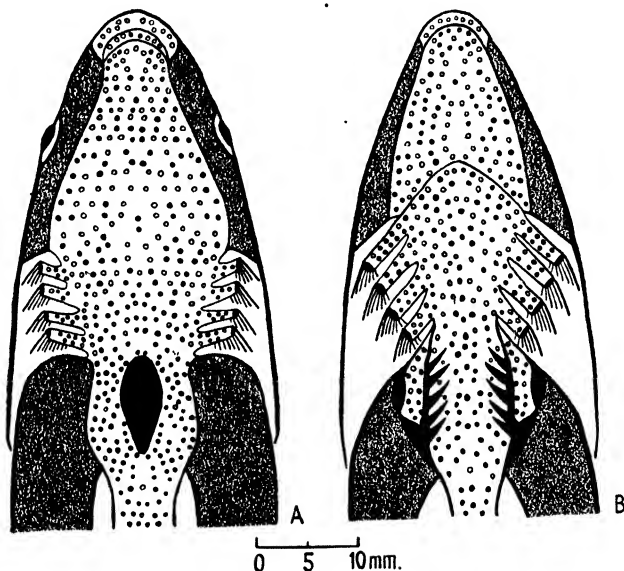
In *Gobio* the two convex 'palatine cushions' before mentioned (p. 119) are richly supplied with taste-buds and pyriform mucus-secreting cells, the latter being specially concentrated along the sides of the crypt which separates the two cushions. The sub-epithelial connective tissue is richly vascular, and contains numerous collagen fibres and nerves. An attempt was made to demonstrate the action of these cushions experimentally by first anaesthetizing a fish and then presenting it first with a piece of gravel, and then with food (meat) of similar size held close to the mouth. Every time without exception the particles would be sucked in with the respiratory current and on touching the cushions the gravel would be immediately rejected, but the food particles would be retained, at least for some time. It is thus clear that the cushions act as food selectors and, at the same time, act as an efficient barrier to the passage of all other material entering the mouth, allowing it to be immediately rejected. The significance of such a mechanism is self-evident when one recalls the bottom-feeding, mud-grubbing habits of the fish. The highly specialized gustatory sense of both *Cyprinus* and *Gobio*, in which they also

resemble *Mulloides auriflamma* (Al-Hussaini, 1946), is clearly an adaptation to the bottom-feeding habit.

The Pharynx

The Anterior Pharynx

In all three fish the mouth merges gradually, with no abrupt change, into the pharynx. The mucus-secreting cells become gradually more numerous and tend to become concentrated along the sides of the crypts so that, in



TEXT-FIG. 4. Diagrammatic representation of the distribution of taste-buds and mucus-secreting cells in the fore-gut of *R. rutilus*. A, the roof; B, the floor of the mouth, pharynx, and oesophagus. Taste-buds open circles (o), mucus-secreting cells solid (●).

transverse section, they have the appearance of multicellular glands (Pl. II, fig. 5). In some places they become huddled together and stratified, and encroach upon the undifferentiated epithelial cells which, although multi-layered high up on the sides and on the crests of the folds where mucus-secreting cells are few, are reduced to a single layer at the bases of the crypts. This arrangement resembles that found in other fishes I have previously investigated and described.

Taste-buds remain numerous, especially in *Gobio* and *Cyprinus*; in the lateral portions of the anterior pharynx of the latter fish especially they form an almost unbroken sequence (Pl. I, fig. 4), while on its roof there is the palatal organ of Valatour, with its acknowledged gustatory function (Herrick, 1904).

The foregoing account concerning taste-buds and mucus-secreting cells conforms closely to the generalization which I drew concerning the pharynx in teleosts (Al-Hussaini, 1946), namely, that it is a region concerned with gustation as well as mucus production, irrespective of the kind of diet of the fish, and that the relative extent of these two functions is slightly altered when the food contains a great amount of silt, as, for example, in *Scarus* and *Mul-loides*. An additional feature is a high concentration of both taste-buds and mucus-secreting cells on the gill-rakers in the Cyprinids (Pl. II, fig. 6). Presumably the mucus serves to trap food particles detected by the taste-buds and so prevent their ejection with the respiratory current, but an attempt to demonstrate this experimentally led to indefinite results since direct observation of this region of the pharynx could only be made at the expense of normal respiratory movement.

Concerning the sub-epithelial structures of the anterior pharynx in general the following noteworthy points may be mentioned. The stratum compactum is thinner than in the mouth, in fact in some places it is difficult to detect. In the tunica propria there are numerous striated muscle fibres (Pl. I, fig. 4). In general these are arranged singly and transversely but they may run in various directions and may be assembled in groups. A striking feature of these muscle-fibres is that in several places they are closely associated with the stratum compactum, while they also invade the cores which support the taste-buds. They presumably serve to mobilize these structures as well as the general mucosa of the pharynx during the processes of mastication and swallowing. The large adipose cells already noted in the submucosa of the floor of the mouth extend also into the pharynx and are found in addition in the roof but not at the sides of this cavity.

The submucosa of the pharyngeal roof is much thicker in *Cyprinus* than in either *Gobio* or *Rutilus*, thus accounting for its more 'fleshy' appearance, while its nerve-supply is very rich—a feature obviously to be correlated with the profusion of taste-buds found in this species.

The Posterior Pharynx

The mucosal folds of this region are high; they branch and anastomose freely. Mucus-secreting cells abound (Text-fig. 5c) and attain their maximal development in this region, being of the saccular type (Text-fig. 3g, h).

Taste-buds are still numerous, although in *Rutilus*, but not in *Gobio* or *Cyprinus*, they become much less abundant posteriorly.

The horny pad, characteristic of the roof of the posterior pharynx, consists of an especially thick stratified epithelium (Pl. III; fig. 8). Of the three easily recognizable zones the basal (B.Z.) consists of a single layer of cells following the sinuosities of the tunica propria underlying it. The middle zone (M.Z.) comprises large polygonal cells, the cytoplasm of which stains deep red with eosin and crimson with Mallory's triple stain, while the nuclei remain pale and show nucleoli. The cell membranes are much thickened, and fine cytoplasmic bridges connecting neighbouring cells together are clearly visible.

The number of layers of cells forming the superficial zone (s.z.) is about equal to those of the middle zone in *Rutilus* but is smaller in *Gobio* and larger in *Cyprinus*. Although the individual cells of the superficial zone also have thick membranes they show a highly characteristic and very irregular outline as if crumpled and distorted. They take a bright-red colour with eosin and stain orange with Mallory: thus the superficial zone is sharply contrasted to the one below it. The nuclei are retained but in a very degenerate condition, although Curry (1939) described and figured similar cells in the common carp as 'enucleated'. The superficial cells are shed from the surface either singly or in groups. The superficial zone does not occur on the anterior or posterior extremities of the pad, which exhibits here a somewhat attenuated form. The staining reactions of this zone are those characteristic of horn, and the hardness of the pad is in direct proportion to its thickness, being greatest in the herbivorous and least in the carnivorous example.

Numerous coarsely striated muscle-fibres are found at the base of the pad, which presumably would be capable of effecting some adjustment of its position. The stratum compactum is very thin in the posterior pharynx and is composed of only a few compact collagen fibres.

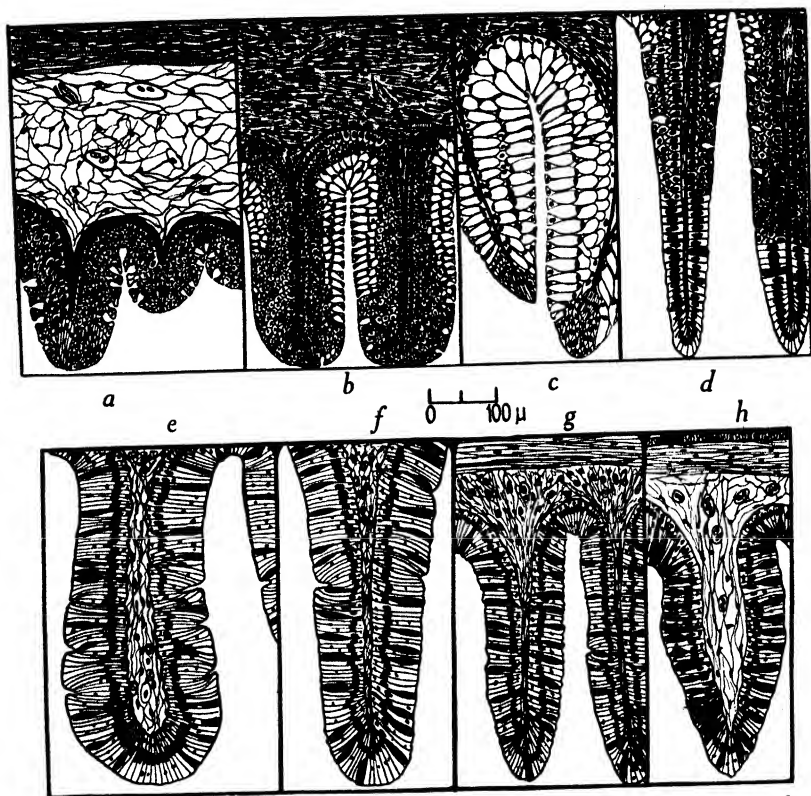
The histology of pharyngeal teeth has already been described in *Mulloides* (Al-Hussaini, 1946) and the present fishes exhibit similar features except that their teeth are firmly fixed to the underlying pharyngeal bones.

The Oesophagus

The oesophagus is customarily defined in higher vertebrates as the transitional region between the pharynx where the glottis opens and the stomach, so on this basis it must be regarded in the Cyprinids as the region of the gut extending from the horny pad and pharyngeal teeth to the point where the intestinal epithelium begins, or rather to the constriction (pyloric sphincter) at the commencement of the intestine (Pl. III, fig. 9). Only the ventral and lateral aspects of the oesophagus are covered by serosa, immediately beneath which is a dense layer of striated muscle. This single muscle layer, although of a general circular character, is not precisely transverse to the longitudinal axis of the oesophagus which is itself oblique. At the junction between oesophagus and intestine the oesophagus undergoes a pronounced thickening and forms a pyloric sphincter (M.C.). A pyloric sphincter has been recorded in the goldfish by McVay and Kaan (1940), but no reference was made to it by either Rogick (1931) in the minnow or Curry (1939) in the common carp. The submucous connective tissue is very compact and contains many coarsely striated muscle-fibres which run haphazardly in various directions and which extend up the very base of the mucosal epithelium, being separated from it only by a very thin stratum compactum consisting of a very few collagen fibres.

The mucosal folds of the anterior end of the oesophagus resemble, in general, those of the posterior pharynx; their branching system, giving rise to primary and secondary folds, has already been described for the common carp by Edinger (1877) and Oppel (1896). Taste-buds are still plentiful in *Gobio*

and *Cyprinus* but are very scarce in *Rutilus*, while mucus-secreting cells of the saccular type are present in all three. At the caudal end of the oesophagus the mucosal folds become very much deeper and the stratified squamous epithelium characteristic of the crypts and bases of the folds changes to a



TEXT-FIG. 5. Schematized drawings of individual mucosal folds from various parts of the alimentary canal of *R. rutilus*. Only the goblet cells, among the various types of mucus-secreting cells, are shown in black. (a) buccal cavity; (b) anterior pharynx; (c) posterior pharynx, lateral wall; (d) oesophagus; (e) intestinal swelling; (f) second limb of intestine; (g) third limb of intestine; (h) rectum.

columnar epithelium as the crest of the fold is approached (Text-fig. 5d). This columnar epithelium contains mucus-secreting cells of the goblet type only and no taste-buds.

The arrangement just described is the reverse of that found in the common carp by Curry (1939), while in the minnow Rogick (1931) described a mixture of epithelial types in the mucosal folds of the oesophagus.

From the foregoing study and from knowledge gained from three fish studied earlier (*Scarus*, *Mulloides*, and *Atherina*, Al-Hussaini, 1945, 1946;

1947a) it is possible to reach two important conclusions concerning the morphology of the oesophagus in teleosts. Firstly, the mucosa changes gradually from a stratified epithelium with saccular mucus-secreting cells to a columnar epithelium with mucus-secreting cells of the goblet type. Secondly, its muscular coat consists of a dense layer of striated fibres circular in direction. In *Atherina* alone a layer of longitudinal fibres occurs internal to the circular layer, and in all the examples studied the submucosa, and even the tunica propria, always contains scattered muscle-fibres. The pyloric sphincter is formed by the thickening of the circular layer.

Thus the oesophagus is distinguished from the pharynx on the one hand by its first mixed and then purely columnar epithelium, and from the intestine on the other by the type and arrangement of its musculature (in fishes with a stomach the multicellular 'cardiac' glands may serve as a posterior diagnostic boundary). It is hoped that this account may help in clearing away the vague definitions so much in vogue. As thus defined the oesophagus of the Cyprinids here studied shows no adaptation to feeding habits apart from the fact that taste-buds are more extensive in the anterior region in *Gobio* and *Cyprinus* than in *Rutilus*.

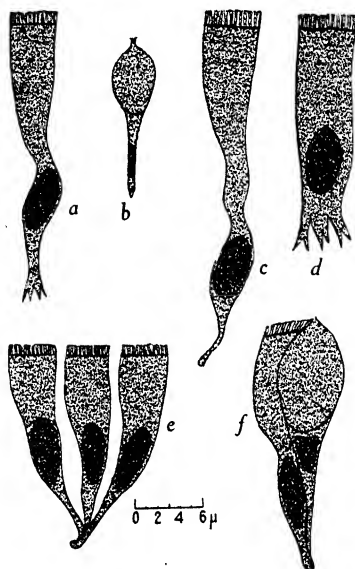
Immediately posterior to the horny pad the pneumatic duct opens into a short dorsal diverticulum formed by two major folds of the oesophagus, at which point the mucosal cells of the oesophageal folds become columnar. The duct, near the opening, is ensheathed by a thick layer of fibrous tissue; to the outside of this is a dense layer of longitudinal striated muscle-fibres which is in turn surrounded by a similar layer of circular fibres. Presumably these fibres regulate the opening and closing of the aperture of the duct.

The Intestine

The intestine is lined by a simple columnar epithelium comprising only two principal types of cells but containing also two or three other subsidiary cell-types which will be dealt with in detail below. The more common of the two principal types is that usually described, even by recent authors (e.g. Maximow and Bloom, 1945, and Clark, 1945), as 'columnar' or 'cylindrical' on account of their appearance in normal micro-sections. As will be shown later, the shape thus seen refers to a part of the cell only; the other part, not easily seen in sections, is variable according to the position of the cell in the mucosal fold. Following the lead recently given by Baker (1944) the term 'absorptive cells' is used in the present paper, thus putting emphasis on their function rather than their shape.

The true shape of these cells is revealed by the maceration method of Goodrich (1942). It will be seen (Text-fig. 6) that the majority are **not** straight but, in addition to a general tapering towards the base, each cell is bent at least once. These bends do not all occur at the same level but interlock with similar bends in neighbouring cells and this, presumably, serves to strengthen the epithelium as a whole. The nuclei are variously disposed within the basal portions of the cells; their presence always involves a bulging of the cell

outlines, which by interdigitating with the swollen nuclear portions of neighbouring cells further serves to strengthen the epithelial sheet. The basal terminations of the epithelial cells, which may be knob-like (*c, f*) or finger-like (*a, d*), serve to anchor the cells to the subjacent tissues. Sometimes several cells may be anchored together by this means (*e*). The free border, at least in the intact epithelium, seems to be continuous from one cell to another.



TEXT-FIG. 6. Drawings of isolated cells from the intestinal epithelium of *R. rutilus*. (*a*), (*c*), (*d*), and (*e*), absorptive cells; (*b*) goblet cell; (*f*) absorptive and goblet cells in apposition.

Only two previous attempts to study the intestinal cells of fishes by a maceration method appear to have been made. The first, by Edinger (1877), working with the common carp, reveals simply an inverted conical structure ending in a filamentous process, and shows neither the perinuclear swelling nor the terminal nodules or processes. The second attempt was by Stirling (1884), using the herring as material. His figures of the cells between the oesophagus and the cardia of the stomach resemble Edinger's except that the filamentous basal process is shorter.

Macklin and Macklin (1932) put forward the idea that the spaces between the filamentous processes of the absorptive cells are not separate intercellular spaces but one continuous space, filled with fluid which freely communicates with the tissue fluid in the cavities of the sub-epithelial core, either by diffusion through the permeable basement membrane or possibly by actual minute perforations. Thus what may be termed the 'excretory

area', or the surface area across which substances may diffuse from the absorptive cells to the body fluids, is enormously increased by this tapering of the basal portions of the epithelial cells. In *Gobio* the cells are shorter than in *Rutilus* and *Cyprinus* and hence the 'excretory area' is accordingly smaller.

The second of the two principal cell-types found in the intestinal epithelium is the mucus-secreting cell. These are typical goblet cells (Text-fig. 6b, f) which attain their maximum concentration in the rectal epithelium (Text-fig. 5e-h)—a fact which, surprisingly enough, has hitherto escaped the attention of investigators. This high concentration of goblet cells in the rectal mucosa obviously facilitates defaecation.

Other cellular structures which may be observed within the intestinal epithelium include small lymphocytes which, although commonest within the fluid-filled intercellular space around the bases of the absorptive cells, may migrate between them to within a short distance of the free border. A second type consists of granular cells (absent from *Gobio*). These are particularly abundant in the submucosa and, although they may invade the basal part of the epithelial layer, they do not migrate so near to the free border as do the lymphocytes. (This is in marked contrast to the condition observed in the three fish studied earlier, Al-Hussaini, 1945, 1946, 1947a.) They may also be found in the connective tissue between the two muscle layers and beneath the serosa and will be discussed in more detail in Part II of this paper. Finally, a third type of cell, of infrequent occurrence, comprises cells, pear-shaped in outline, which reach the free border and are recognizable by their cytoplasmic inclusions and spheroidal nucleus. Their precise significance is obscure and they will be dealt with in more detail in a subsequent paper.

Regarding the sub-epithelial tissues, the stratum compactum, better developed in *Gobio* than in *Rutilus* or *Cyprinus*, lies in close proximity to the epithelium and not in the middle of the submucosa as in the Salmonidae (Oppel, 1896; Greene, 1912). If the view that the stratum compactum acts as a girdle, checking undue distension of the intestine (Baecker, 1940), be accepted, then the intestines of *Rutilus* and *Cyprinus* will be more distensible than that of the carnivorous *Gobio* since their strata compacta are weaker, a feature that may be correlated with the more bulky nature of their food.

The muscular coat consists of an inner circular and an outer longitudinal layer. A transition from striated to non-striated muscle occurs in the circular layer immediately caudal to the pyloric sphincter and in a roach 7 cm. long is complete by 2.2 mm. beyond the choledochal duct. The unstriated fibres first appear internal to the striated fibres, and the transition is accomplished gradually, so that, in progressively caudal transverse sections, the striated layer becomes gradually thinner and the non-striated wider.

Using the common carp as an example of teleosts, Li (1937) described a thin layer of longitudinal fibres next to the submucosa. Using the same technique on the Cyprinids now being considered it has not proved possible to demonstrate a continuous sheet of longitudinal fibres in this position, although in a few places isolated patches of such fibres were seen. A few points remain to

be dealt with concerning the intestine. The choledochal duct unites with the pancreatic duct and then immediately opens into the intestinal swelling, not, as described by McVay and Kaan (1940) in the goldfish, on the summit of a papilla, but simply between two mucosal folds, their epithelium becoming continuous with that lining the ducts.

In the rectum the submucosa is richly vascular, and since the mucosal folds are shallower and broader than in the intestine the sub-epithelial cores are more extensive. The anal outlet is lined by a stratified epithelium containing numerous pyriform mucus-secreting cells like those of the skin, from which, indeed, it is distinguished only by the absence of 'Kolbenzellen' characteristic of the latter. The circular muscle thickens to form an anal sphincter. In all the three cyprinids the luminal surface of the epithelium of the intestinal swelling is commonly indented (Text-fig. 5e) so that the surface is lowered forming a pit-like depression. A central cell extends in a straight line from the pit to the submucosa, but the cells around it are curved so that they form collectively a sort of 'nest'. These cells have a more or less uniform breadth, their nuclei are not compressed, and their cytoplasm stains but feebly; the cells around the 'nest', however, are much compressed, their nuclei elongated, and their cytoplasm stains more deeply. These 'nests' of cells also occur in the second limb of the intestine, though less commonly, while in the third limb of the intestine and rectum they are rare. Their significance is unknown; they cannot be homologous with primordial gastric glands because they are post-pyloric in position.

DISCUSSION

Biologists practically agree that the roach is typically omnivorous, the gudgeon predominantly carnivorous, and the mirror carp mainly herbivorous; the small quantity of vegetable material taken in by the gudgeon and of animal food eaten by the mirror carp merely serving to 'balance' the diet (cf. Kyle, 1926). The following discussion is based on this assumption.

None of the three types possesses teeth on either jaws or palate, but all have well-developed pharyngeal teeth firmly affixed to the modified fifth gill arch which oppose a horny pad carried on a special masticatory process of the basi-occipital. Mastication is thus effected in the pharyngeal region, as it is in scarids and labrids which crush respectively coral and molluscan shells, and hence does not obviously interfere with respiration since it takes place posterior to the respiratory part of the gill apparatus.

The form of the teeth also shows a correlation with the type of diet. Thus the carnivorous *Gobio* has biserial hooked teeth, the omnivorous *Rutilus* has uniserial teeth, some of which are hooked, while the herbivorous *Cyprinus* has three rows of teeth converging towards each other forming a stud-like 'molariform' prominence. Both the relative overall size of the pharyngeal jaws as well as of the muscles which actuate them are best developed in *Cyprinus* and least in *Gobio*, while the hardness of the horny pad is also in direct relationship to the amount of plant food ingested.

Of the sixty-odd species of teleosts previously examined and described (Al-Hussaini, 1947b), most of the herbivorous species have serrated, 'incisiform' oral teeth and very weak pharyngeal ones. They are thus capable of nibbling the plants by means of their front teeth and the food enters the mouth in a ready-macerated form. In the grey mullets and also, according to Wier and Churchill (1945), in the gizzard shad (*Dorosoma cepedianum*), the pylorus forms a 'gizzard' which triturates diatoms and the like. Fishes living on plant food or hard food have thus acquired one mechanism or another which enables them to mince their food in preparation for digestion; therefore the correlation of the morphological features of the alimentary tract with the feeding habits is complicated by the genetical factor. For example, the carnivorous *Gobio* is more closely comparable to the herbivorous *Cyprinus* than the latter is to a herbivorous acanthurid like the doctor fish, *Acanthurus sohal*, which possesses oral teeth, a stomach, and pyloric caeca. When *Gobio* and *Cyprinus* are compared with each other, however, several significant points of difference become apparent, as, for example, the stronger 'molariform' teeth in *Cyprinus* and the longer intestine. It is consequently not always possible to draw hard-and-fast lines between the several groups of feeders based on certain characteristic features, because fishes, during their progressive evolution, have become variously adapted to the conditions surrounding them, diverging gradually from each other in the process. An excellent example is afforded by the three herbivorous species *Acanthurus sohal*, *Cyprinus carpio*, and *Mugil auratus* belonging to three distinct families. The plant food on which these fishes graze is comminuted by the serrated 'incisiform' oral teeth in the first, by the pharyngeal teeth in the second, and by the gizzard-like pylorus in the third species—three different structures, but all, in fact, fulfilling a similar function. On the other hand, *Gobio* can be compared with *Mulloid*es and *Cyprinus* with *Mugil*. The first two are carnivores and the last two are herbivores, but all four are bottom-feeders, stirring up the mud and seeking their food from it, and all lose their oral teeth.

Although the cyprinid gut is simple and its relative length (cf. p. 121) is smaller in *Rutilus* and *Cyprinus* than for the average omnivorous or herbivorous fish, yet estimations of the mucosal area (made here for the first time) show that this is not small relative to that of mammals, and that variations in the relative length of the gut may well be compensated by variations in the mucosal area (cf. p. 123 for a fuller discussion of these matters).

Turning now to histological features we find that the abundance of taste-buds is rather to be correlated with the way in which the fish secures its food than with its nature. Thus when a fish has to select its food from mud (*Gobio* and *Cyprinus*) or sand (*Mulloid*es), or to single out living coral from amongst inorganic material (*Scarus*), an efficient gustatory sense is imperative, especially around the entrance to the mouth cavity, and does not depend on the nature of the nutritional substances: and taste-buds are much more numerous in the species named than in the free-feeding, omnivorous *Rutilus* or the

plankton-feeder *Atherina*. Nevertheless, taste-buds are quite common structures within the mouth in *Rutilus*.

The histology of the teleostean intestine is one of the simplest among vertebrates. In the Cyprinids under consideration the intestinal epithelium shows no special features beyond the formation of the special cellular 'nests' referred to above (p. 134); indeed, multicellular glands are characteristically absent from the teleostean intestine except in some of the Gadidae (Jacobs-hagen, 1937). A spiral valve is present in *Chirocentrus*, while vestiges of it remain in some of the Salmonidae and in *Gymnarchus* (Goodrich, 1909); the typhlosole-like structure found in *Scarus* may well be peculiar to this genus (Al-Hussaini, 1947*b*). A rectal gland is never present in teleosts but pyloric caeca frequently are (cf. Al-Hussaini, 1946 and 1947, for a fuller discussion of these structures).

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SUMMARY

1. The mouth is larger and more protrusible in *Gobio* and *Cyprinus* than in *Rutilus*, features which may be associated with the mud-grubbing, bottom-feeding habits of the two former fish, while the arrangement of the adductor muscles in *Rutilus* enable it to close its mouth more quickly than they and hence it can rapidly snap up its free-moving prey.

2. The selection of food is largely by taste in *Gobio* and *Cyprinus*, and hence both are richly supplied with taste-buds from lips to oesophagus, special concentrations being present on barbs around the mouth. *Rutilus*, on the other hand, augments taste by sight. Taste-buds are accordingly more restricted in distribution and less abundant where they do occur. They are fewer on the lips; there are no barbs.

3. The relative development of the pharyngeal masticatory apparatus (horny pad, pharyngeal teeth) bears a direct relationship to the amount of plant food in the diet, i.e. it increases in the order *Gobio*, *Rutilus*, *Cyprinus*.

4. The mucus-secreting cells attain their maximum development in the

pharynx in all three fishes, as is usual amongst teleosts (cf. Al-Hussaini, 1947a, p. 278) and are thus effectively placed to lubricate the food, irrespective of its nature, at the very commencement of its journey through the gut.

5. Gill-rakers carrying taste-buds and mucus-secreting glands are present in all three fish, but they are exceptionally short in *Gobio* for a bottom-feeding fish. This may be compensated for to some extent by a pair of food-selecting palatal cushions.

6. All three fishes are stomachless, the short oesophagus joining the pharynx directly to the pyloric sphincter. The loss of the food-holding capacity of the stomach is compensated for by the swelling of the first limb of the intestine.

7. The intestinal tube is longest and its looping most complex in *Cyprinus*, and shortest and with the simplest looping in *Gobio*. Four parts are recognized in the intestinal tube, not by external features, but by their mucosal foldings and certain histological characters.

8. An estimation of the mucosal area shows that the absorptive area of the intestinal epithelium is practically equal in the three species when related to the weight of the fish. This ratio has here been called the 'mucosal coefficient'.

9. The intestinal epithelium comprises two principal histological cell types, viz. the absorptive cell and the goblet cell.

10. The internal surface across which food substances pass from the cells to the tissue fluids is greater in *Cyprinus* and *Rutilus* than in *Gobio*, owing to the greater length of the absorptive cells.

11. In order to be valid, estimations of the relative efficiency of the fish intestine should take into account the relative length of the gut (R.L.G.), the mucosal coefficient (Q.M.), and the length of the absorptive cells.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. *R. rutilus*, showing mucosal folds of roof of mouth, pharynx, oesophagus, and intestinal swelling.

Fig. 2. Transverse section of the buccal cavity of *Cyprinus carpio*. $\times 22$. Note the abundance of taste-buds (T.B.) all over the cavity except on the dorsal surface of the maxillary valve (MX.V.).

Fig. 3. Tangential section of the lower lip of *Cyprinus carpio*, showing scores of taste-buds in cross-section. $\times 85$.

Fig. 4. Transverse section of the epithelium lining the lateral part of the roof of the anterior pharynx of *Cyprinus carpio*. $\times 190$.

PLATE II

Fig. 5. Transverse section of the anterior pharyngeal region of *Cyprinus carpio*. $\times 26$.

Fig. 6. Transverse section of the anterior pharynx of *Cyprinus carpio* passing through a gill arch. $\times 45$.

Fig. 7. Pharyngeal 'jaws' of the three Cyprinids. $\times 2$ approx. (a) the left 'jaw' of *R. rutilus* showing six teeth, medioventral aspect; (b) both 'jaws' of *R. rutilus* from the dorsal aspect; note asymmetry of teeth; (c) the right 'jaw' from the same specimen as (a), medioventral aspect; (d) the left 'jaw' of *Cyprinus carpio*, lateral aspect; (e) the right 'jaw' from the same specimen as the previous one, lateral aspect; (f) both 'jaws' of *G. gobio*, ventral aspect. In (a), (b), (c), and (f) the anterior end is pointing downwards.

PLATE III

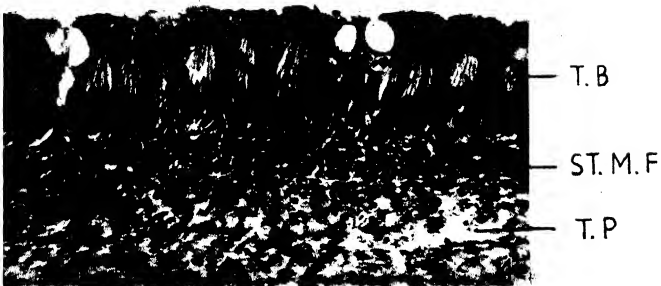
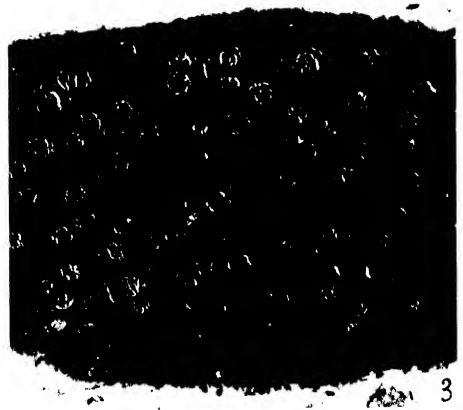
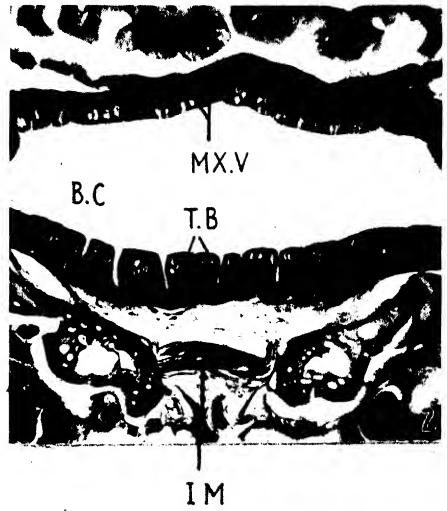
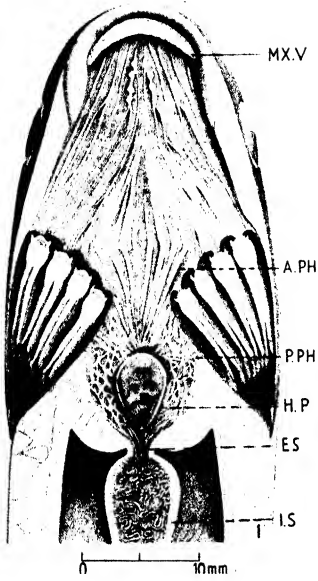
Fig. 8. Transverse section of the horny pad of *R. rutilus*. $\times 70$. (a) drawing to show the cellular types composing the three zones of the pad.

Fig. 9. Transverse section through oesophagus (ES.) & intestinal swelling (I.S.) of *R. rutilus*. $\times 20$.

Figs. 2-6, 8, and 9 untouched photomicrographs; figs. 2-6 and 9, fixed Boling's fluid stained H & E; fig. 8, fixed Boling's fluid, stained Mallory's triple.

LIST OF ABBREVIATIONS USED IN THE ILLUSTRATIONS

A.B.	air-bladder.	M.P.	masticatory process of basi-occipital.
A.PH.	anterior pharynx.	M.S.C.	mucus-secreting cells.
AN.	angular.	M.Z.	middle zone.
AR.	articular.	MD.AD.	mandibular portion of adductor mandibulae.
B.C.	buccal cavity.	MX.	maxilla.
B.M.	body musculature.	MX.AD.	maxillary portion of adductor mandibulae.
B.Z.	basal zone.	MX.V.	maxillary valve.
C.A.	coeliaco-mesenteric artery.	OP.	opercular.
D.A.	dorsal aorta.	P.D.	pneumatic duct.
D.O.	dilator operculi.	P.PH.	posterior pharynx.
DT.	dentary.	PH.C.	pharyngeal cavity.
ES.	oesophagus.	PL.	palatine.
GH.	geniohyoideus.	PMX.	premaxilla.
G.R.	gill raker.	POP.	preopercular.
H.P.	horny pad.	R.A.B.D.	retractor arcus branchialis dorsalis.
I.S.	intestinal swelling.	R.L.	rostral ligament.
IM.	intermandibularis.	S.Z.	superficial zone.
IN.	intestine.	SH.	sternohyoideus.
I.O.	interopercular.	SOP.	subopercular.
L.	ligament between maxilla, premaxilla, and dentary.	ST.M.F.	striated muscle-fibre.
L.A.P.	levator arcus palatini.	T.B.	taste-bud.
LV.	liver (hepatopancreas).	T.P.	tunica propria.
M.C.	muscularis circularis (forming sphincter).	TH.G.	thymus gland.
M.L.	muscularis longitudinalis.	TZ.	trapezius.



Observations on Hypotrichous Ciliates: The Genera *Stichotricha* and *Chaetospira*

BY

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INTRODUCTION

WHILE examining the empty cells of certain pondweeds, I became interested in two hypotrichous ciliates that occurred there with some regularity and often in considerable numbers. The one that drew my attention first was a loricate organism that I identified as a species of *Chaetospira* Lachmann (1856). The other was a non-loricate form which was much less modified for a restricted habitat, and migrated from cell to cell even when adult. This second organism answered to the description of *Stichotricha* Perty (1852), although its morphology did not precisely accord with that of any of the species recognized by Kahl (1932).

Whereas the free-swimming scavenging Hypotricha, such as *Euplotes* and *Stylonychia*, have been exhaustively studied, little has been recorded of the behaviour and reproduction of the cell-inhabiting species, although the restriction on their movements makes them comparatively easy to observe for long periods at a time. The following notes, though fragmentary, may serve to fill some gaps in our knowledge; and they may also help to clear up some taxonomic confusion. After a brief historical survey, I propose to deal first with *Stichotricha*, since it is the more comparable with 'normal' hypotrichs: more precise information as to its structure, life-history, and behaviour undoubtedly helps in the interpretation of the highly modified *Chaetospira*.

HISTORY

The confusion in the nomenclature of these genera is almost impossible to resolve. *Stichotricha* was first described in 1852 by Perty, 'lancelet-shaped with the extensible anterior end narrow, flat, drawn out and carrying the peristome. On one side of this is a row of large obliquely-standing cilia.'

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Although his description was so incomplete that the ciliate 'could have been a *Loxodes* or *Amphileptus*' (Lachmann, 1856), Perty's name *Stichotricha secunda* for the type species has been retained. He placed it in the Oxytrichina. Four years later Lachmann (1856) described two species of a new ciliate genus *Chaetospira*, *C. mülleri* and *C. mucicola*, both of them freshwater and loricate. Of this genus he wrote, 'when extended the proboscis (Rüssel) forms more than one turn of a spiral and the first membranelle is longer and stronger than the rest'. He placed *Chaetospira* near *Stentor* but realized that it was related to *Stichotricha*. In 1862 Strethill Wright described, but did not figure, another species of *Chaetospira*, which he named *C. maritima* on account of its marine habitat. A third freshwater species, *C. remex*, was described by Hudson (1875); but this, together with Bolton's *C. cylindrica*, recorded in 1878, was relegated by Savile Kent (1880) to the genus *Stichotricha*. Gruber (1879) had already said that *Stichotricha* and *Chaetospira* were identical, and his *S. urnula*, described in 1883, certainly is very closely related indeed to *Chaetospira* and possibly forms a bridging species between the two genera. Entz (1884) again identified *Chaetospira* and *Stichotricha* and said that his newly described *S. inquilinus* was none other than *C. mülleri*. Möbius (1888) recorded *C. maritima* from the Kiel Canal and figured it. Meanwhile eight other species of *Stichotricha* had been described. Some of these are possibly species of the genus *Chaetospira*.

Another complication was introduced by Sterki (1897), who, while giving the most complete description as yet of *Chaetospira mülleri*, seems not to have known of this genus. He called his ciliate *Spirotricha paradoxa*, and he appreciated the differences between it and *Stichotricha*. Kahl (1932) returned Sterki's *Spirotricha*, together with a variety *S. paradoxa univacuolata* Illowaisky (1913), to the genus *Chaetospira*, species *mülleri*. In the same work Kahl recorded *C. entzi* as another bridging species between *Chaetospira* and *Stichotricha*, the thirteen described species of which he reduced to eight.

MATERIAL AND METHODS

Chaetospira was first found by me on algal encrustations at water level on the sides of a shallow glass jar of pond water in which were growing *Lemna*, *Cladophora*, and *Riccia*. The sources of the material were not recorded. Later collections were made from ponds in or near London. These never yielded cysts as did the first batch, and the *Chaetospira* were always found in the dead cells of *Lemna trisulca* or *Riccia fluitans*. Once they occurred in the root of *Lemna minor* and once in the dead cells of some unrecognizable plant debris.

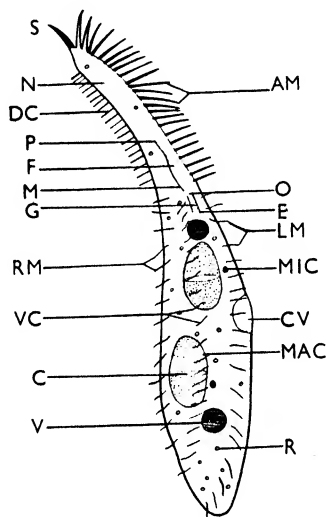
Stichotricha was found inhabiting the empty cells of leaves of *Lemna trisulca* and roots of *L. minor*. These two species of *Lemna*, collected over a period of 18 months, were kept in tongue jars in the laboratory.

Most of the observations were made on living material and consisted in watching behaviour, division, and, in *Chaetospira*, lorica formation. Certain individuals of this genus could be watched for several weeks; but *Stichotricha*

rarely stayed in the same cell for more than a day. However, this was long enough for all the stages of asexual reproduction to be followed.

In feeding experiments the animals were placed in pond water enriched with bacteria and small undetermined flagellates, or in a carmine suspension.

For cytological purposes *Lemna* leaves or roots having a fair ciliate population were fixed in alcoholic Bouin or Champy, Zenker or Schaudinn and stained with Heidenhain's iron haematoxylin, haematein (after Dobell),



TEXT-FIG. 1

Delafield's haematoxylin, or haemalum by the Vicelle method. This method I had direct from Professor Hovasse, who recommended it for demonstrating micronuclei: after fixation wash overnight in tap-water, rinse in distilled water, and stain in a very weak aqueous solution of haemalum for 24 hours. Feulgen following Champy was frequently used.

STICHOTRICHA INTERMEDIA N. SP.

Trophic Phase (Text-fig. 1)

This species, like others of the genus, has a contractile, flexible, spindle-shaped body, drawn out anteriorly into a proboscis (N), usually bent but never spiral, bearing along its left border a row of powerful adoral membranelles (AM). The whole animal is slightly spirally twisted especially when extended in feeding (Text-fig. 2, fig. 1). The terminal membranelle is larger and set apart (S). Ventrally there run three longitudinal rows of cilia curving posteriorly to the right (RM, LM, VC). At the base of the proboscis lies the mouth (O) and to the right of it a hyaline ectoplasmic membrane (M). There are two

macronuclei (MAC), and the contractile vacuole (CV) is dorsal and on the left, roughly half-way along the length of the body.

In the adult of this species, length varies from 40 to 170 μ : when extended about two-fifths is occupied by the proboscis. It is characterized by two rows of dorsal hair-like cilia (DC), all the other species, according to Kahl (1932), having three rows. There are in this species, in *S. secunda* (for a full account of ciliation in *S. secunda* see Stein, 1859), and probably in all others, a row of fine, stiff, seta-like cilia supporting the ectoplasmic membrane on the right of the peristome. These form a parabolic curve (P), and, following Sterki's nomenclature for similar structures in *Spirotricha* (= *Chaetospira*, 1897), they may be called the paroral cilia. The membrane, together with its cilia, runs back to the mouth from the point where the proboscis is bent. Endoral cilia (E) line the gullet.

The pellicle is very thin. There is no differentiation into ectoplasm and endoplasm, but the cytoplasm of the proboscis is hyaline and contains only a few greenish, refringent granules (R). These are more numerous in the main mass of the body, where also lie the nuclei, contractile and food vacuoles (V). The contractile vacuole, which at diastole projects on the left dorsal surface, contracts every 15–20 seconds—at more frequent intervals in young than in older individuals. The anus is mid-dorsal and the creature emerges from the sheltering cell just enough to allow the faecal material to be deposited outside (Text-fig. 2, fig. 1 A).

Perty (1852) did not mention the nucleus. Kahl (1932), in his monograph on Ciliates, says of the nucleus of *Stichotricha* only that it is in two pieces. Obviously this refers to the macronucleus. The two lie in tandem, each an ovoid structure with its long axis parallel with that of the ciliate. In the living organism the macronucleus appears as a refringent greenish body. About half-way along its length is a transverse cleft. In the preserved ciliate the macronucleus is made up of a chromatin network surrounding a number of vacuole-like spheres (cf. Willis, 1942, and Wenrich, 1929b). Embedded in the side of, or somewhere near, each macronucleus is a micronucleus. This stains densely and uniformly in interphase, but is not visible in the living animal.

Behaviour

The species of *Stichotricha* under consideration is intermediate in its habits between the freely swimming *S. secunda* and the loricate *S. socialis*. Unlike the former it may remain in one particular cell for several days, but never inhabits the same cell permanently as does *S. socialis*.

When feeding, the animal is extended (Text-fig. 2, fig. 1), the proboscis projecting beyond the aperture of the sheltering cell and bending to one side, the posterior end of the body being braced to one or more sides of the cell wall by the posterior cilia.

When disturbed the animal withdraws rapidly to the inmost corner of the cell, moulding its shape to fit against the wall. At the same time the proboscis

is contracted and thickened but never retracted into the body (cf. *Chaetospira*). Withdrawal is brought about by the adoral membranelles. The ciliate usually emerges again quickly and continues feeding. It may, however, remain withdrawn for a longer time, especially if environmental conditions are unfavourable, e.g. increased temperature or gradual desiccation. These conditions, amongst others not yet determined, also cause the migration of the animal to a new site. The behaviour of the ciliate during migration is the same as that of the swarmer after asexual reproduction, and is dealt with in the following section.

Stichotricha usually feeds on bacteria and other small organisms. Occasionally it will swallow larger organisms such as coloured flagellates. *Chlamydomonas* spp. measuring $7\ \mu$ in diameter were fed to these *Stichotricha*, and even though the flagellates were as broad as the proboscis (i.e. three times the diameter of the gullet), they were ingested (Text-fig. 2, fig. 3). As there are no records of feeding methods in *Stichotricha*, a brief account based on my observations may be of interest.

A feeding current is promoted by the adoral membranelles and is sufficiently powerful to bring in particles from some considerable distance.

Small particles, about $1\ \mu$ in diameter, are ingested indiscriminately if they get carried to the food groove. Larger particles, $7\text{--}10\ \mu$ in diameter (width is the important dimension), are undoubtedly selected. Particles of carmine and faecal debris of this size are always rejected. Flagellates are always accepted.

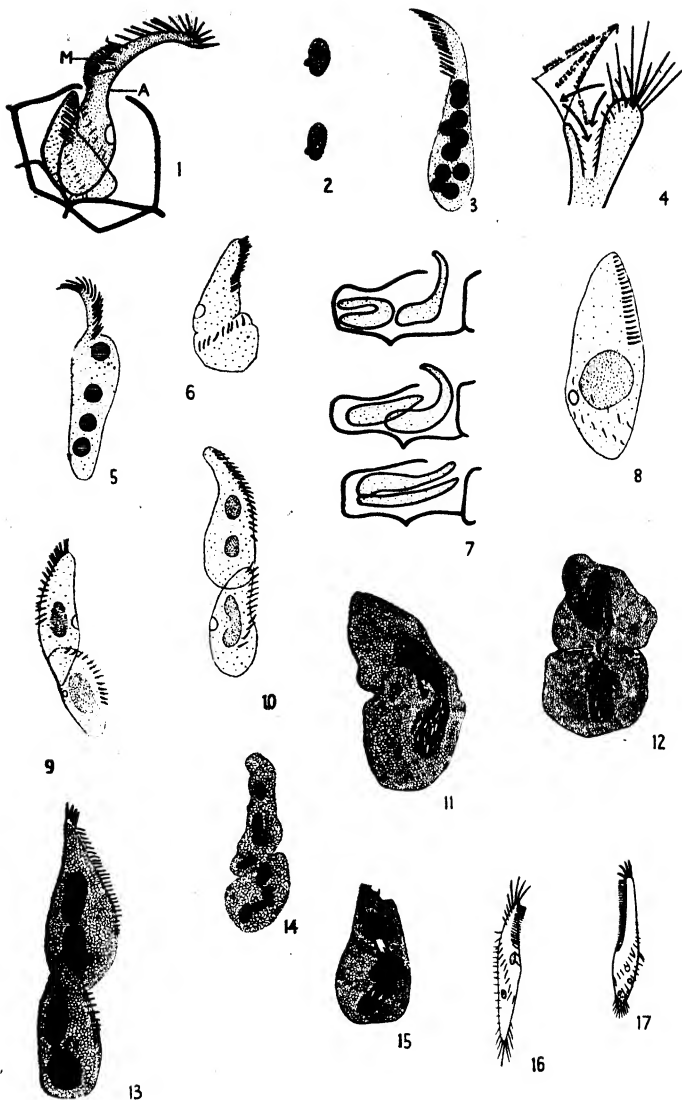
Playing an important role in selection is the membrane and its paroral cilia. These are so placed in relation to the row of adoral membranelles that a V-shaped food groove lies between them, its floor formed by a strip of proboscis cytoplasm running the length of the membrane and back to the mouth. Normally the membrane is held stiffly, and curves outward gently from the base of the food groove directing the current. In doing this it plays a passive role (Text-fig. 2, fig. 4).

When a large edible particle is brought into the food groove, the membrane, moved by the parorals, closes over it and appears to push it down into the gullet. With very large edible particles, such as the *Chlamydomonas* already mentioned, strenuous gulping movements of the cytoplasm take place as in *Stokesia* (Wenrich, 1929a). While the monad is trapped by the membrane and is revolving in the food groove at the base of the peristome, the cytoplasm within the body surges forward and surrounds it. The combined efforts of the displaced cytoplasm and the membrane finally bring about ingestion (Text-fig. 2, fig. 5). After this the cytoplasm that had piled up at the base of the proboscis returns to its normal position.

Rejection of large particles is accomplished by spasmodic jerks of the membrane.

Asexual Reproduction

Preparatory stages. Animals about to divide by binary fission have not necessarily reached a maximum size. Whereas the ciliate in Text-fig. 2, fig. 1.



TEXT-FIG. 2. *Stichotricha intermedia* n.sp.

(Except where otherwise stated, drawings, made with aid of camera lucida, are of living specimens $\times 360$.)

1. Left side view of adult feeding. Contracted position also shown. M, ectoplasmic membrane. A, position of anus. 2. Nuclear apparatus of adult. $\times 800$. Feulgen. 3. Specimen that has fed well on algae. 4. Diagram of proboscis seen end on at the bend, showing feeding currents. 5. Displacement of cytoplasm in swallowing a large food particle. 6. Onset of cytoplasmic fission. 7. The products of fission after separation but before migration. Freehand. 8. The two macronuclei have fused prior to fission. 9. After fission but before separation, each daughter ciliate with a single macronucleus. 10. The single macronucleus of each daughter dividing. 11. The fusion macronucleus and two micronuclei dividing during binary fission. Haemalum. $\times 800$. 12. The fusion macronucleus and two micronuclei of each daughter dividing. Haemalum. $\times 800$. 13. The two macronuclei of each daughter ciliate dividing. Haemalum. $\times 800$. 14. Each daughter ciliate with four macronuclei. Haemalum. 15. The two macronuclei of a daughter ciliate dividing after separation. Haemalum. $\times 800$. 16. *S. simplex*, after Kahl. 17. *S. gracilis*, after Möbius.

has grown to $96\ \mu$ before dividing, that in fig. 6 has divided when only $52\ \mu$ in length. Other workers have found that the rate of multiplication in ciliates may be independent of size (Harding, 1937, and Chatton and Beauchamp, 1923). Adolph (1931) came to the conclusion that 'when the processes which have to do with age come to a certain point, fission occurs regardless of how much body substance is present'. In the case of the organism described here it may be that the size of the containing cell in some way determines division. The onset of fission is marked by the withdrawal of the ciliate and consequent cessation of feeding, whereas other Hypotricha are active during division. Beating of the posterior adoral membranelles continues.

Cytoplasmic fission. Fission begins on the oral side just behind the peristome and extends by an oblique line across to the dorsal side behind the contractile vacuole (Text-fig. 2, fig. 6). The posterior half acquires new organs, whereas the anterior half retains those of the parent, as in *Euplotes harpa* (Wallengren, 1901). The new membranelles appear before cleavage is complete, but in what order I am unable to say. In addition a contractile vacuole is working in the posterior half by this time. While the line of cleavage deepens, the dividing ciliate rotates from time to time and may slowly expand and contract. When fission is complete, the two resulting ciliates remain one behind the other for some time, their membranelles beating actively. About 10 minutes later the posterior individual or swarmer moves up towards the opening of the cell and lies alongside (Text-fig. 2, fig. 7) or in front of the anterior individual, and it is difficult to distinguish one from the other. The whole process occupies about 2 hours.

Fate of the swarmer. The two individuals produced as a result of binary fission may remain together within the same cell, providing there is sufficient room for both to protrude and feed. If this happens, then the anterior one extends its neck and begins to feed shortly after fission, whereas the swarmer spends a preliminary period within the enclosing cell, extending and retracting, rotating and 'exploring'. After this it partially emerges and commences feeding. This remaining together of the products of fission sometimes results in groups of four individuals; but these are independent and not alined as in *S. socialis* (Gruber, 1879).

Migration and settlement. It is more usual for the swarmer to migrate after division. Before doing so it makes excursions to the aperture of the cell, pushing its sister ciliate back. These exchange movements continue for some time, the swarmer seeming to become bolder and emerging tentatively through the cell opening. Finally, it squeezes the broader posterior end of its body through and escapes. Its behaviour now depends upon local conditions.

If there is plenty of *Lemna* and it comes into contact with this, it will crawl over the plant in a persistent and exploratory manner, searching for a possible settling place. It does this by means of the membranelles and the few body cilia. The crawling is a jerky progression in straight lines, alternating with sudden backing movements brought about by a reversed beat of the membranelles. In backing the front part of the body is bent on the rest and

then straightened suddenly so that the ciliate points in a new direction in which it now proceeds. The front end is continually poking into crevices and empty cells. Sometimes these will be entered. Feeling all round the inside of the cell, using the larger anterior membranelles, projecting the anterior end through the aperture of the cell as if testing the nature of the surrounding water, and then backing and twisting round inside, the swarmer seems to test thoroughly the new abode. It may remain here or leave and begin the process of trial again. No feeding is done during this migratory period. As with *Hypotricha* in general and the swarmers of the *Peritricha*, the ciliate at this stage is markedly thigmotactic.

If, however, there is not much *Lemna*, and the swarmer swims straight out into open water after binary fission, then its behaviour is different. The swimming is continued. This is a rapid forward spiralling movement brought about by the membranelles and by the fact that the body is lop-sided owing to the disposition of the curved proboscis. As a result of the spiralling a much larger volume of water is explored. Although the movement is usually forward, reversals are not infrequent, and the bending of the front end, with subsequent change in direction, results sooner or later in some solid surface being contacted. Then the crawling and exploring movements begin.

The duration of this free-swimming phase varies from one to many hours (cf. *Chaetospira*).

Cytology. The behaviour of the macronuclei can be followed in the living ciliate. Fusion of the two macronuclei takes place before the cytoplasm cleaves (Text-fig. 2, fig. 8). Shortly after fission has started, the fusion nucleus divides into two, one of which passes forwards into what will be the anterior individual, while the other takes up its position in the swarmer (Text-fig. 2, fig. 9). Before fission is completed each macronucleus divides again. The products of this division, however, do not become the definitive nuclei of the new individuals, for they are seen to undergo the initial stages of a further division before the daughter ciliates separate. This division may be completed before or after separation (Text-fig. 2, figs. 14 and 15). Presumably the four macronuclei now in each product of division fuse two by two to form the two adult macronuclei, the line of fusion being represented by the cleft characteristic of hypotrichous nuclei.

The behaviour of the micronuclei can be followed only in stained material. While the fusion macronucleus is dividing, the two micronuclei divide to form four and then eight. The fate of these is obscure for only two persist in each daughter ciliate. Perhaps as in *Kahlia simplex* (Horvath, 1936) some of them disintegrate and do not participate in the final stages of fission (Text-fig. 2, figs. 11, 12, and 13).

While dividing, the chromatin of the macronucleus is in the form of beaded threads (Text-fig. 2, fig. 14), short and thick in the early and late stages, long and attenuated in the middle stages. These threads are so numerous and crowded together that it is impossible to count them. The same is the case with the micronuclei; and here extremely small size adds to the difficulty.

TABLE I. Showing Chief Distinctive Structural Features of Nine Stichotrich Species
(.. indicates no record)

SPECIES	<i>secunda</i>	<i>marina</i>	<i>aculeata</i>	<i>socialis</i>	<i>saginata</i>	<i>gracilis</i>	<i>opisthotonoides</i>	<i>simplex</i>	<i>intermedia</i> n.sp.
FOUNDER .	Perty	Stein	Wrzesniowski	Gruber	Möbius	Möbius	Smith	Kahl	Froud
DATE .	1852	1867	1870	1880	1888	1888	1897	1930	1944
HABIT .	Solitary	Solitary	Solitary	Social	Solitary	Solitary	Solitary	Solitary	Solitary
HABITAT .	<i>Chara</i> & <i>Myriophyllum</i>	<i>Ulua</i>	<i>Sphagnum</i>	Flooded meadows	<i>Ulua</i>	..	Old infusions	..	<i>Lemna</i>
LORICA .	Absent	Absent	May or may not be present	Gelatinous	Absent	Absent	Absent	Absent	Absent
LENGTH IN μ .	130-200	180-260	100	200	200	100	55	85-120	40-170
PROBOSCIS .	Bent	$\frac{1}{4}$ body length	Straight $\frac{1}{4}$ body length	Bent $\frac{1}{4}$ body length	Thick	..	Bent dorsally	$\frac{1}{4}$ of body length	Bent $\frac{2}{3}$ body length
FRONTAL CIRRI	3-4	..	5	..	5	None
ROWS OF BODY CILIA .	4	4 ridged	4	4	4	2	..	2	3
LENGTH OF DORSAL CILIA IN μ	20	7	7	5

Systematic Position

Of the eight species of *Stichotricha* described by Kahl (1932), *S. simplex* (Text-fig. 2, fig. 16) agrees most closely with the one described here (see Table I). Both have three rows of cilia on the narrow, spindle-shaped body and the proboscis occupies a third of the body length. Both are non-loricate and, though widespread, are not profuse. The size range of *S. simplex*, 85–120 μ in length, is comparable with that for my species, but it has five frontal cirri whereas mine has what might be called a frontal cirrus, but this is really the first membranelle, which sticks out horn-like as does that in *S. secunda*. The dorsal cilia of *S. simplex* measure 7 μ as compared with 5 μ in the new species. These are only small differences, and it might be considered that my species is identical with *S. simplex*; but it does not seem wise to give it the same name until more is known about them both.

Again it is possible that the species described here is identical with *S. gracilis* Möbius, sketched by him in 1888. This species also shows reduction of body ciliation (Text-fig. 2, fig. 17) although Kahl (1932) attributes this to oversight in observation. No account of *S. gracilis* was given. If it does have only two rows of body cilia (see Table I), then it has gone farther in the process of cilium-reduction than my species, which is intermediate between it and the more typical species. Here again, until more is known about *S. gracilis*, it seems best not to identify mine with it.

S. opisthotonoides (see Table I) is also so incompletely described that it is impossible to say whether I have been dealing with it rather than with a new species. Like mine it is freshwater, solitary, naked, and has a bent proboscis. Its small size (55 μ) might well be accounted for by the fact that the specimens measured by Smith (1897) were all young ciliates, in which case they would agree with the species described here.

On these grounds a new species is proposed for this *Stichotricha*, and because several of its characters are intermediate between those of other species of the genus it is called *S. intermedia*.

The following is the diagnosis of *Stichotricha intermedia*:

Systematic position. *Stichotricha intermedia* n.sp. (Spirotricha, Hypotricha, Oxytrichidae).

Description. Solitary; non-loricate; length of adult 40–170 μ , two-fifths of which is occupied by a bent proboscis; three rows of body cilia; dorsal cilia 5 μ in length, two rows.

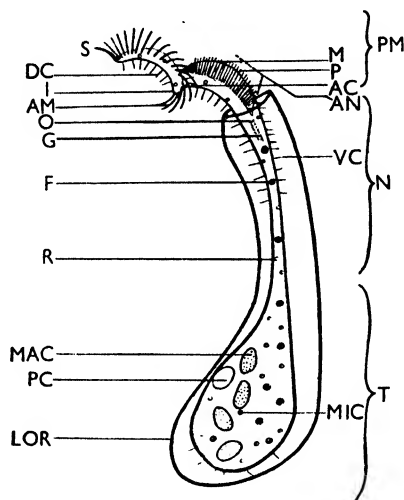
Habitat. Lemna.

Locality. London, England.

*CHAETOSPIRA MÜLLERI LACHMANN**Trophic Phase* (Text-fig. 3).

Chaetospira is a flask-shaped organism varying in length from 60 to 200 μ . When feeding, the neck of the flask is drawn out into a spiral (PM), which may form as much as a third of the length of the body, and runs in an

anti-clockwise direction. On this proboscis are borne the powerful adoral membranelles (AM). Of these the first few are stouter and more widely separated than the rest. The first (s) in particular is very conspicuous, differing from the others in size, appearance, and behaviour. In addition to the cilia of the membranelles there are accessory peristomial cilia (vc), stiff, sensory dorsal cilia (dc), and a small undetermined number of body cilia. Opposite the adoral membranelles on the left-hand margin of the peristome and running from the first twist of the spiral back to the mouth (o) is a thin



TEXT-FIG. 3

ectoplasmic membrane (M) supported by a series of stiff paroral cilia (P). The mouth lies at the base of the proboscis and leads into a gullet (G) which penetrates the neck. This and the proboscis are composed of hyaline cytoplasm containing a few of the refringent granules which in a well-nourished specimen fill the base of the flask-shaped body (T). This contains the contractile vacuole (PC), food vacuoles (F), and the nuclei (MAC, MIC). There may be another contractile vacuole (AC) on the proboscis, left and dorsal to the adoral membranelles, on the first turn of the spiral. As in *Stichotricha* the pellicle is very thin and the body is plastic, without any real distinction between ectoplasm and endoplasm. The anus is far forward on the proboscis, dorsal, to the left and a little way behind the first turn of the spiral (Text-fig. 4, fig. 18).

Hitherto the nucleus of *Chaetospira* has not been adequately described. In Sterki's words, 'Two almost globular endoplasts were seen not very distinctly.' Kahl, in 1932, said that the nucleus of *C. mülleri* is in two separate pieces. Both these remarks refer to the macronucleus. Now my species of *Chaetospira* has its macronucleus in rounded pieces, usually four, as in *Gastrostyla*

(Weyer, 1930). But as Lachmann, the discoverer of *C. mülleri* (with which species I think I am dealing), did not mention the nucleus, there is no justification for setting up a new species until the cytology of the genus has been studied.

In the living animal nuclei cannot be seen. Feulgen-stained specimens (Text-fig. 4, fig. 20) show the macronucleus to be made up of a chromatin network surrounding a number of vacuole-like spheres as in *Stichotricha*. The number of macronuclei in trophic individuals varies from two to eight

TABLE II. Showing that Macronuclear Number is independent of Body Length in *C. mülleri* (fixed material)

No. of macronuclei	8	6	5	4	3	2	1
Body length in μ of individuals	31 54	32 42 57	30 32 42 54 69 128	32 41 54 72 91 111 132	40 69	35 72	35 65

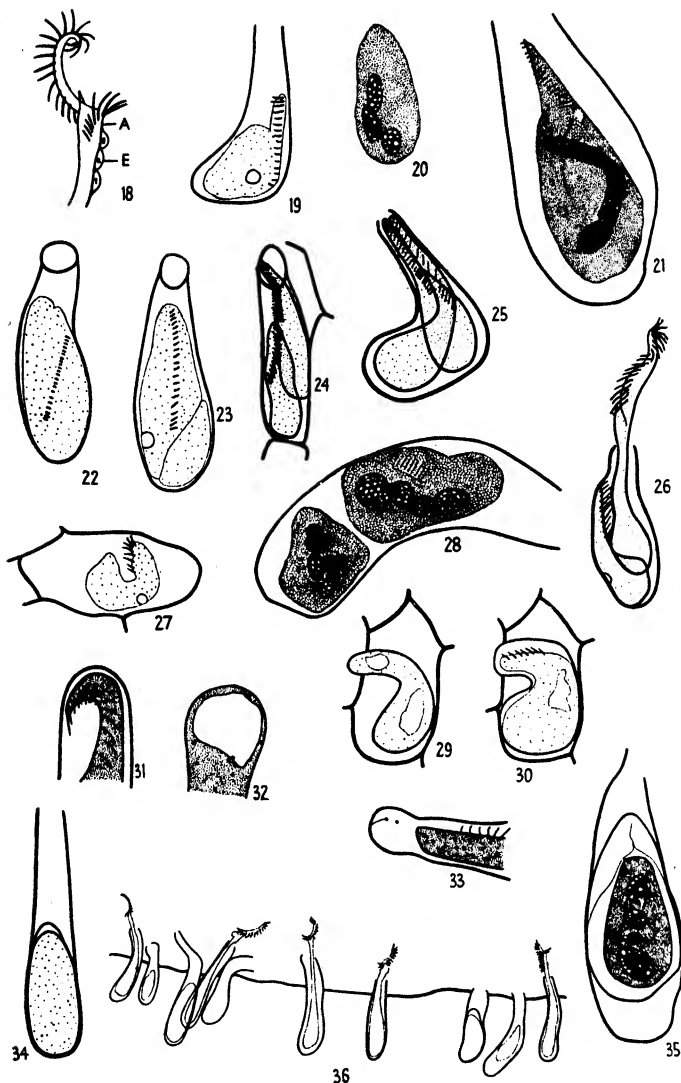
(cf. *Urostrongylum contortum* Kahl (1932)). There is no correlation between the number of macronuclei and the size of the individual (see Table II). This may be accounted for by one or more of the following assumptions:

1. Endomixis might be occurring in these apparently ordinary individuals. Against this is absence of disintegrating macronuclei.
2. Mitosis might be completed before cytoplasmic fission starts. Against this is the rareness of division figures; but supporting it are the individuals of all sizes with five to eight nuclei.
3. In many ciliates, e.g. *Stentor*, the adult macronucleus is the product of several 'Placenten' (Bělař, 1926). This might account for the large number of macronuclei in some of the small ciliates, and for what looks like the fusion of macronuclei in pairs in a few cases.

The micronucleus is usually single, but at times two or more have been seen. It lies near or in a notch in the side of one of the macronuclei (Text-fig. 4, fig. 21). In interphase it is densely and uniformly stained. Consequently when it lies above or below a macronucleus it cannot be seen. This may account for the variation in number recorded above. On the other hand this might be due, as in the case of the macronucleus, to precocious mitosis.

Feeding

Chaetospira feeds on the same kinds of food and in the same way as *Stichotricha*. I have seen it ingest *Bodo*, diatoms, and considerable lengths of algal filaments. Sterki (1897) observed this and noted also that the posterior adoral membranelles are responsible for rejection. This I have not seen. The



TEXT-FIG. 4. *Chaetospira mülleri*

(Except where otherwise stated, drawings, made with aid of camera lucida, are of living specimens $\times 360$.)

18. Diagrammatic sketch of proboscis, ventral view, showing three successive positions of a faecal pellet about to pass out through the anus A. E, ectoplasmic membrane. 19. Retraction of proboscis in a contracted animal. 20. Nuclear apparatus of adult. $\times 580$. Feulgen. 21. Fusion macronucleus prior to fission. $\times 580$. Haemalum. 22. Dormant phase prior to fission. 23. Cytoplasmic fission. 24, 25, and 26. Different appearances of the products of fission after separation but before migration. Freehand. 27. Swarmer exploring a new site. 28. The nuclear picture immediately after fission. Haemalum. $\times 800$. 29. Formation of lorica: vacuolation. 30. Same: withdrawal. 31. Neck of new lorica before it is open. Freehand. 32. Adding new length of neck to lorica: vacuolation. 33. Cyst. 34. Cyst. 35. Same, stained Heidenhain's iron haematoxylin. $\times 580$. 36. Group of feeding, resting, and encysted individuals. $\times 72$.

membranelles can be seen moving only during extension or retraction of the proboscis and at certain times during asexual reproduction. At other times their rate of beating is so rapid that they appear motionless. I could see no differential beating of the posterior ones such as one would expect if they were the rejecting agents.

In a large, fully extended, feeding *Chaetospira*, food vacuoles are formed every 20 seconds. If cysts are ingested, the contents are utilized and the empty cases egested. The colour of a diatom disappears 10 minutes after ingestion, and *Bodo* is immobilized in less than 1 minute.

Asexual Reproduction

Preparatory stages. Individuals about to divide by binary fission are large and have their cytoplasm packed with greenish granules. The first sign of division is the withdrawal of the proboscis and rounding of the anterior end of the now pear-shaped body. Because the adoral zone is contracted, no feeding is done and the creature is sluggish, but the posterior membranelles continue to move though slowly and discontinuously. When not moving they are closely pressed to the body. This dormant period lasts for several hours (Text-fig. 4, fig. 22).

Cytoplasmic fission. Fission takes place as in *Stichotricha* (Text-fig. 4, fig. 23). All the dividing ciliates that I watched were in loricas and not easy to observe on account of the disposition of the sheltering cells, so that I was not able to determine how or when the ciliation of the posterior individual is laid down. It is this posterior half that acquires new organs, whereas the anterior half retains those of the parent. While the two new ciliates are still attached by a strand of cytoplasm dorsally, the membranelles of both begin to beat, but no attempts are made at this stage to emerge and feed. After some time separation is effected and the two inhabitants of the lorica now roll round one another, frequently changing places (Text-fig. 4, figs. 24 and 25).

Escape of the swarmer. The behaviour of the two individuals is different. The membranelles of the posterior individual or swarmer are less well developed than those of the anterior. They appear ragged and beat haphazardly so that the swarmer makes jerky movements. It will come up to the base of the neck of the lorica, stick out its now pointed anterior end, and then withdraw rapidly to the back end of the lorica. When it does this the anterior individual is first displaced from the neck end and retires, then moves forward again when the potential swarmer withdraws. The movements of the anterior individual with its clear-cut membranelles are more regular; and after a time it extends its proboscis at intervals through the lorica neck and feeds (Text-fig. 4, fig. 26). Feeding is interrupted when the swarmer makes its forward exploratory movements. Sometimes both are forward together (Text-fig. 4, fig. 25).

Not for long do the two remain in one lorica. They are too cramped and it is impossible for them both to feed. The swarmer comes more and more frequently to the lorica neck, pushing the other back: it makes more and more

frequent essays at extending its proboscis through the lorica mouth, and finally squeezes out through the neck and swims away. The anterior individual is now left in possession of the lorica. It immediately proceeds to feed and carries on as a typical trophic *Chaetospira*.

Migration and settlement. The swarmer acts as a distributive phase. It is still unlike the adult in that the proboscis is relatively undeveloped, with few membranelles and not spirally twisted but bent over to one side as in *Stichotricha*. The size of the swarmer varies with that of the parent, but an average length of $46\ \mu$ was obtained from fifteen specimens. On leaving the parent lorica the behaviour of the swarmer is very similar to that of *Stichotricha* (Text-fig. 4, fig. 27).

The duration of this free-swimming phase varies. In one case, escape from the parent lorica was followed by immediate contact with a leaf of *Lemna*, discovery of an empty cell 5 minutes after, and its exploration and acceptance 10 minutes after this. In another case, the free-swimming ciliate was watched for half an hour before it finally settled.

I must remark here upon the striking resemblance between this distributive organism and the corresponding stage in *Stichotricha*. Morphologically they are identical except for small details in ciliation and in the nature of the nucleus. The *Chaetospira* swarmer is never free for such long periods as that of *Stichotricha*. As regards adaptation to a loricate existence, *Chaetospira* has gone much farther than the other ciliate, and the adult is considerably modified accordingly. But here is an example of a 'young' stage, retaining during its development the facies of a less-specialized genus of the family. Possibly this resemblance has been responsible for some of the confusion of the two genera, observers having mistaken swarmers of *Chaetospira* for free-swimming individuals of *Stichotricha*. In view of this some revision of stichotrich species seems necessary.

Cytology. Owing to the innumerable greenish granules present in the cytoplasm during division, it is impossible to follow nuclear behaviour in the living organism. Consequently my observations have had to be made on preserved material. This is unsatisfactory for the nuclear pictures are so variable that I am as yet at a loss to explain many of them. All I can say at present is that I believe the following to happen during binary fission:

The macronuclei fuse. (Text-fig. 4, fig. 21). The fusion nucleus divides into two, four, eight, or more, usually. Cytoplasmic fission separates an anterior individual with four macronuclei from a posterior one with eight (Text-fig. 4, fig. 28). These eight fuse in pairs forming the four adult macronuclei. The fusions are not necessarily simultaneous. Moreover, they can take place before or after the daughter cells have separated. The micro-nuclear cycle has not been followed.

Lorica Formation.

The adult lorica (Text-fig. 4, fig. 36). This is flask-shaped, with its base embedded in an empty plant cell and its neck protruding from the opening.

It is thin and perfectly smooth. I can find no evidence for its being sticky, except perhaps when it is first laid down. The accumulation of debris around the neck of the flask, described by Möbius (1888) and Sterki (1897), is due to the method of feeding: for in *Stichotricha*, where there is no lorica but the same mode of feeding, the debris collects round the opening of the cell sheltering the animal. In both these ciliates rejected solid matter tends to be deposited where the quickly travelling water-current meets the static pond water. This is outside the ambit of the membranelles at the base of the proboscis and, in *Chaetospira*, on a level with the mouth of the lorica.

Möbius (1888) called the lorica chitinous, though he makes no mention of chemical tests to support his statement. Tests for chitin are difficult to make on such a small object. With picro-nigrosin and Mann's methyl blue eosin, I got positive results: the lorica stained blue in each case. Yet with iodine in potassium iodide followed by zinc chloride the result was negative. A negative result was also obtained with Millon's reagent, suggesting that the lorica is not protein.

Origin of the lorica. The lorica is laid down in the first place by the swarmer. This, as soon as it has found a suitable settling place, becomes inactive, appears to withdraw its membranelles and cilia, rounds off the pointed anterior end, and undergoes a peculiar process of vacuolation. Just before this there are streaming movements in the cytoplasm. Then the greenish granules with which it is well stocked pass to the periphery and large vacuoles appear throughout the body (Text-fig. 4, fig. 29). This leads to inflation and the ciliate becomes half as large again. By this means the limits of the new lorica are determined, for it must be larger than the animal it shelters and allow for growth. Complete immobility follows for about 5 minutes. Then there is a slight oscillating movement of the greenish granules. Meanwhile the vacuolation has increased, the lorica becomes very definite on the surface of the cytoplasm, and, 10 minutes or so after, the animal seems to be composed almost entirely of one enormous vacuole and appears dead. Only 5 minutes after this, vacuolation is reduced, movement of the cytoplasm recommences, and withdrawal from the lorica begins (Text-fig. 4, fig. 30). This starts in the region of the membranelles, which now reappear and, apparently better developed, become active. The contractile vacuole can now be seen again. Roughly an hour after the swarmer first becomes free it is established. That there is some close connexion between the granules and lorica-formation is obvious, but I am unable to say how the material in the granules reaches the surface and gets deposited there. In each case, however, some of the granules are left behind adhering to the newly formed lorica neck when the ciliate withdraws. Moreover, although normally rare in the peristome region, at this time they are numerous there.

Opening of the mouth of the lorica takes a variable time and I am unable to say whether it is accomplished by mechanical or chemical means. The ciliate moves backwards and forwards in the lorica, using its adoral membranelles. As it moves forwards, the proboscis creeps along the neck of the

lorica to which its dorsal surface is closely applied. When it gets to the closed end it bends over, still keeping in close contact. At this stage the ciliate seems to be pushing against the closed end with the back of its neck (Text-fig. 4, fig. 31). This lasts only a short time, and is followed by return to the base of the flask. Then the cycle of movements is repeated. Finally a break through is made and the ciliate can extend its proboscis and feed.

Additions to the lorica. Unlike the ciliates *Lagenophrys tatersalli* Willis (1942) and *Folliculina ampulla* Fauré-Fremiet (1932), where no additions are made to the adult lorica, *Chaetospira* adds to its from time to time. More frequent additions are made in the young than in the old ciliate. In all its stages the process of addition is similar to that of lorica formation by the swarmer, except that when inflation takes place the front end of the vacuolating *Chaetospira* emerges as a sphere from the mouth of the lorica (Text-fig. 4, fig. 32). The surface of this sphere is continuous with the neck of the lorica. After some time, during which presumably the surface of the sphere is converted into lorica material, the cytoplasm within the sphere reorganizes itself into the peristome of the ciliate. This now withdraws from the sphere which shrinks a little (Text-fig. 4, fig. 33). An opening is made at the distal pole of the sphere and it contributes a new length of neck to the lorica. Old specimens have very long necks. The rest of the surface of the ciliate contributes a new layer within the old lorica wall.

Encystment

Cysts occurred only in one batch of material, where they were discovered in encrustations at water-level in the winter of 1944-5. The first stages of encystment were not seen, nor was excystment.

Living cysts are pear-shaped and measure from 35 to 55 μ in length and 15 to 20 μ in breadth. Each lies in the lorica of the individual which forms it, and has a perfectly smooth wall 1 μ thick, except at the pointed end lying next the neck of the lorica. Here it thickens to 2 μ or more (Text-fig. 4, fig. 34). The contents are in close contact with the cyst all the way round and are finely granular and homogeneous, very different from the cytoplasm of the adult. There are no greenish granules, no cilia, no contractile vacuole or other organs.

The most common number of macronuclei in a cyst is three (Text-fig. 4, fig. 35), but cysts with two to six have been seen. This suggests that nuclear division might take place within the cyst as in *Gastrostyla* (Weyer, 1930) and *Kahlia simplex* (Horvath, 1936). One or two micronuclei are present. These I have not seen dividing. The nuclei have the same appearance as those of the unencysted adult.

No work has yet been done on the factors causing encystment. Since the pH of the medium has been maintained constantly at 7, this factor alone is not responsible. Since cysts were obtained only in winter months, drought and/or lowered temperature might be responsible. Cysts were not obtained the following winter (1945-6), so temperature alone is not the cause.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Professor D. L. Mackinnon, who has given me constant help and encouragement.

SUMMARY

1. The structure, behaviour, and asexual reproduction of a plant-cell-inhabiting hypotrichous ciliate are described.
2. The systematic position of the ciliate is discussed and it is placed in the genus *Stichotricha* Perty as *S. intermedia* n.sp.
3. New information concerning behaviour, nuclear apparatus, and asexual reproduction is given for the loricate *Chaetospira mülleri* Lachmann. Cysts are described for the first time.

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Oogenesis in the Desert Snail *Eremina desertorum* with Special Reference to Vitellogenesis

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INTRODUCTION

ANCEL (1903), working on *Helix pomatia*, was the first to give some attention to the study of oogenesis in Helicids. He described the history of the chromatin, the nucleoli, and the cytoplasmic bodies. In his account of the chromatin cycle, however, this author overlooked the important stages of chromosomal conjugation and simply detailed the stages of chromatin diffusion. Ance's account of the history of the cytoplasmic bodies is very defective owing to the crude technical methods used in cytoplasmic cytology in his time.

Gatenby (1917), in a work primarily focused on the cytoplasmic inclusions of germ cells of *Helix aspersa*, gave subsidiary attention to the chromatin cycle in oogenesis. In fact he did not recognize any of the meiotic prophase except the pachytene 'bouquet'.

Making use of his Flemming without acetic and iron haematoxylin technique, Gatenby could demonstrate and follow satisfactorily the history of mitochondria in oogenesis. He also detected in the ooplasm certain 'Neberkern' bodies (Golgi bodies), but he did not follow their history in any detail. This author could also see in an homogeneous juxtanuclear zone of the ooplasm several blocks of darkly staining material. 'The nature of these structures (Gatenby states) and their connexion if any with mitochondria or Neberkern is unknown to me' (loc. cit., p. 589).

In 1920 Gatenby together with Woodger reinvestigated oogenesis in *Helix* and also studied the same process in *Limnaea* and *Patella*, with the aim of disclosing the mechanism of yolk formation. They observed that especially in *Patella*, the Golgi bodies were plastered on the yolk spheres. This association was taken by the above authors as evidence of the direct metamorphosis of Golgi rods into yolk. Concerning mitochondria, they note that though these elements show growth activities during deutoplasmogenesis, especially in *Limnaea*, much of the evidence is against the view that part of the mitochondrial constituents of the cytoplasm metamorphose into yolk.

Ludford (1921) repeated Gatenby and Woodger's work on *Patella*. He noted that the primordia of the deutoplasmic spheres are deposited under the influence of Golgi bodies and not (as his predecessors thought) that the Golgi bodies change into yolk. Mitochondria, in Ludford's belief, have no direct concern with vitellogenesis.

Brambell (1924) described in the oocytes of *Helix* and *Patella* two distinct categories of deutoplasmic reserve products. He preferred to designate each category according to the cytoplasmic body concerned in its production. Thus he distinguished 'Golgi yolk' and 'mitochondrial yolk'. In the case of *Patella*, he confirmed Ludford's view that the Golgi yolk is formed by, not from, the Golgi rods. In both *Patella* and *Helix*, according to this author, 'mitochondrial yolk' arises through the swelling of mitochondria. Writing about the nature of the Golgi yolk, he favoured the view that it is essentially fatty in both *Patella* and *Helix*.

From the above survey it becomes evident that our knowledge of oogenesis in Pulmonates is still immature. In so far as the chromatin cycle is concerned, it is hardly necessary to say that up till now the full sequence of the Helicid oocytic prophases still abides in the dark and calls for consideration.

Information about the behaviour and role of the two categories of cytoplasmic bodies, viz. Golgi bodies and mitochondria, is more plentiful, but unfortunately contradictory, especially in relation to the part each plays in vitellogenesis. Workers are not agreed as to the exact process by which a Golgi element gives a deutoplasmic sphere. Some consider the process a direct metamorphosis of the one element into the other. Others think that yolk is formed by, not from, the Golgi elements. Also, no agreement is reached as to whether or not mitochondria take a direct part in deutoplasmogenesis. Such a difference of opinion regarding most of the essential points of the mechanism of yolk formation made further study of oogenesis in Pulmonates urgently desirable. Accordingly, Professor K. Mansour (Dept. of Zoology, Fouad I University, Cairo) suggested that the author should undertake a study of the oogenesis in the desert snail *Eremina desertorum*.

MATERIAL AND TECHNIQUE

The desert snail *Eremina desertorum* is a pulmonate Mollusc which occurs in fair abundance in the Egyptian deserts. The material for the present study was brought from Abu-Rawash, a desert district near Cairo.

The desert snail seems to respond readily to environmental conditions. Early in summer (sometimes even late in spring) the animal enters its aestivation period which continues till autumn. Copulation takes place during January. The egg-laying period extends from the middle of February till about the end of March.

As in all Helicids, the hermaphrodite gland of the desert snail lies embedded in the tissue of the liver, at the apex of the visceral hump. The ovotestis was quickly dissected out of the surrounding liver tissue, excised, cut in three or four pieces, and immediately plunged into the fixative.

Most suitable fixation of the nuclear structures was attained by using strong Flemming's mixture for 24 hours. Ordinary Bouin's fluid or urea/Bouin combinations (Ezra Allen's, or Eleanor Carother's; see *Vade Mecum*, 9th edition, p. 319) were also found to be suitable. The nuclear stains used for revealing the chromosomes were iron-haematoxylin and gentian-violet iodine.

For detecting chromatin (strictly speaking the thymonucleic acid thereof), nothing was found more reliable than Wermel's (1927) modification of Feulgen's 'Nuclealfarbung' method.

For the determination of the chromophil nature of the nucleoli, Scott's Ehrlich haematoxylin and Biebrich scarlet method proved unsurpassed. Karyosomes took the haematoxylin, and plasmosomes the orange-red colour of Biebrich scarlet. Recourse was also made to Mann's methyl blue/eosin; Bensley-Cowdry methyl green/acid fuchsin and Pappenheim's methyl green/pyronin techniques. Gatenby's toluidine blue/eosin method was also of great use.

For the demonstration of mitochondria, chromo-osmium fixatives F.W.A., Champy, and Nasonov gave admirable results. Formalin-chrome fixatives of Regaud and Bensley-Cowdry were not very successful with the desert snail's ovotestis; they caused granulation of the mitochondria. The best stain combinations for revealing the mitochondrial material of the desert snail's germ cells were found to be iron haematoxylin/orange G, iron haematoxylin/erythrosin, Altmann's acid fuchsin, and Champy-Kull's acid fuchsin/toluidine blue/aurantia.

The Golgi bodies of the germ cells of the desert snail are preserved by the mitochondrial fixatives, but become most pronounced after post-osmication. Nasonov's modification of Kolatchev's osmium-impregnation technique was by far the best. The Mann-Kopsch and Kopsch's methods also gave good results. The silver-impregnation methods of Da Fano and Cajal, though successful, caused a good deal of shrinkage. Post-osmicated material was either left unstained or stained in Ludford's neutral red.

The demonstration of the Golgi bodies and mitochondria in one and the same cell was found to be an easy matter in all stages of oogenesis of the desert snail. Pieces of the ovotestis were impregnated by Nasonov's technique for 4 days in an incubator at 37° C. This time was just enough for the impregnation of the Golgi bodies, while the mitochondria remained unchanged. Sectioned material was subsequently stained in Altmann's acid fuchsin and

then in aurantia. The Golgi bodies and fat appeared black, the mitochondria red, and the ground cytoplasm golden yellow.

For the detection of fat, Nath's (1934) technique with Sudan III and Scharlach R was tried on fresh and formalin-fixed material and gave good results.

The modern techniques of Baker (1944 and 1946) for the detection of some of the intracellular inclusions by the application of proper chemical procedures on frozen sections of fresh or formalin-fixed material were extremely helpful. His method for the recognition of lipin (acid haematein in conjunction with pyridine extraction) was applied on grown oocytes and enabled the differentiation between mitochondria and proteid yolk.

Lastly, the study of centrifuged oocytes yielded very interesting results.

HISTOLOGICAL FEATURES OF THE OVOTESTIS

The ovotestis of the desert snail is constituted of elongated branched diverticula (Text-fig. 1), opening into the hermaphrodite duct. Although the lumina of some terminal diverticula rarely appear slightly distended, there are no typical globular acini. The ovotestis of the desert snail is therefore more nearly digitate than acinous.

The wall of each diverticulum consists of an outermost layer of connective tissue internal to which comes the germinal epithelium. Each diverticulum during the full swing of germ-cell production contains male, female, and nurse cells (Text-figs. 1 and 2). The female cells and the nurse cells appear close to the wall of the diverticulum whereas the male cells fill its lumen. The earlier stages of the male germ line (the spermatogonia and the spermatocytes) are found nearer to the wall of the diverticulum, while the later stages (the spermatids and the spermatozoa) are found towards the centre of the diverticulum. It must be noted, however, that the concentric arrangement of the successive stages of the male germ line is by no means clear cut and decisive. Sometimes one finds a few spermatocytes lying in the centre of the diverticulum, or a few sperms near the periphery. Towards the blind end of the diverticulum one finds a greater abundance of the earlier male germ cells (Text-figs. 1 and 3), whereas at the open end there appears a greater number of the later stages of the male germ line (Text-fig. 2).

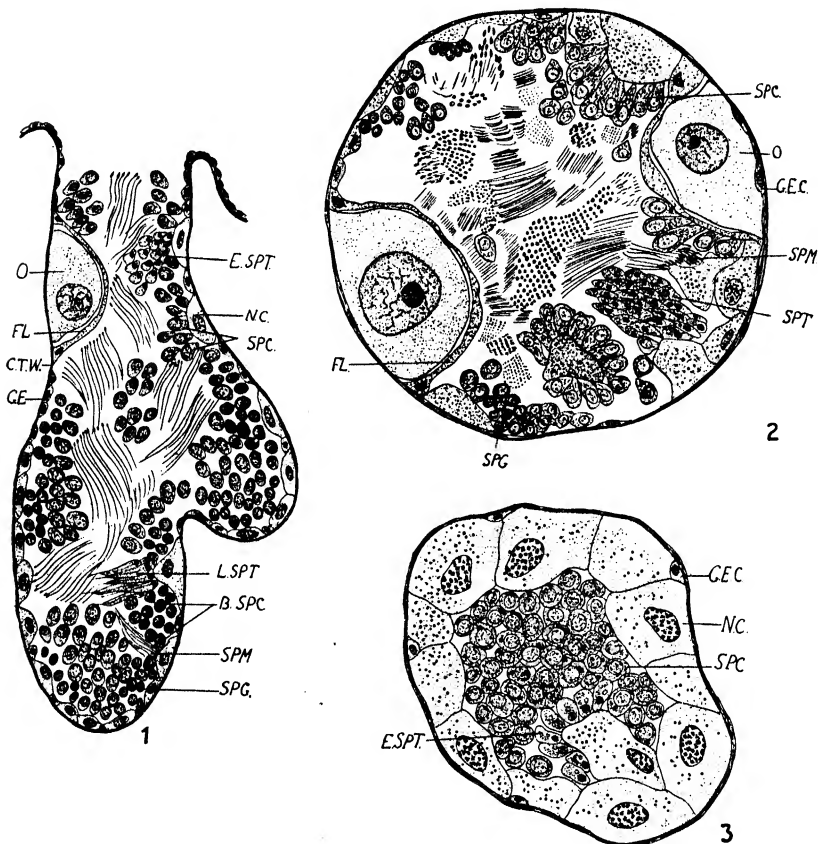
Oocytes may be seen anywhere on the wall of the ovotestis diverticulum, but most frequently nearer to its mouth (Text-figs. 1 and 2). These female elements invariably begin their development underneath a layer of nurse cells and thus remain separated from the male germ cells throughout the whole of their history. A fully differentiated oocyte appears surrounded by a follicle of nurse cells (Text-fig. 2). This follicle gradually dwindles with the progress of oocytic growth and finally disappears when the oocyte is about to pass to the hermaphrodite duct.

Nurse cells are by no means restricted to the oocytic area of the ovotestis diverticulum. They are most numerous and hypertrophied towards the blind ends of the diverticula, where the early stages of the male germ line occur in abundance (Text-fig. 3).

THE GERMINAL EPITHELIUM AND ITS DIFFERENTIATION

Cytological Structure of the Germinal Epithelium

In its undifferentiated condition, the germinal epithelium is constituted of a continuous layer of flattened cells with oval, flattened nuclei (Text-fig. 4).



TEXT-FIGS. 1-3, Sections of the ovotestis diverticula.

Fig. 1, L.S., from a Bouin preparation; $\times 180$. Fig. 2, T.S., near open end, and Fig. 3, T.S., near blind end, both from F.W.A. preparations; $\times 240$.

B.SPC, bouquet spermatocyte; C.T.W., connective tissue wall; E.SPT, early spermatid; FL., follicle; G.E., germinal epithelium; G.E.C., germinal epithelial cell; L.SPT, late spermatid; N.C., nurse cell; O, oocyte; SPC, spermatocyte; SPG, spermatogonium; SPM, sperm; SPT, spermatid.

The chromatin of the nucleus of the germinal epithelial cell is in the form of blocks of irregular shape and unequal size (Text-fig. 4). The number of these blocks is high and also subject to marked variation.

In spite of using a great diversity of fixatives, it was impossible to detect

any connecting bridges between the chromatin blocks of the germinal epithelial nuclei as was described by Gatenby (1917) in the corresponding cells of the garden snail. Ancel (1903) did not describe any such connectives between the chromatin blocks of the germinal epithelial nucleus of *Helix pomatia*. In this respect, therefore, the chromatin of the germinal epithelium of the desert snail is similar to the corresponding chromatin of *H. pomatia*.

The chromatin of the germinal epithelial nucleus takes the basic dyes much better than that of any other cell of the male or female line. Applying Wermel's modification of Feulgen's reagent on sections of the ovotestis of the desert snail, the chromatin blocks of the germinal epithelial nuclei, as well as those of the nurse-cell nuclei, showed the deepest violet colour. The thymonucleic acid content is therefore maximal in the undifferentiated germinal and nurse-cell nuclei.

Nucleoli are absent from the germinal epithelial nuclei. This was ascertained by the application of Wermel's reaction followed by light green. No green colour appeared.

The cytoplasm is not abundant in the germinal epithelial cells. In the majority of cases the nucleus occupies almost two-thirds of the volume of the cell, the cytoplasm the remaining third. Fixing in F.W.A. (diluted by one-third its volume distilled water) and staining in iron haematoxylin and erythrosin, the cytoplasm took the form of an homogeneous reddish mass.

The mitochondrial material of the germinal epithelial cell was also demonstrated by the above F.W.A./iron haematoxylin technique. To one side of the nucleus it was possible to detect a zone consisting of several fine, deep-black, mitochondrial granules embedded in a darkly staining matrix of ground cytoplasm. Also, in the paranuclear zone and rarely in other places of the cell, a few bigger mitochondrial granules may be met with in some, but not all, the cells (Text-fig. 5).

The Golgi bodies of the germinal epithelial cell were not always successfully demonstrated by the F.W.A./iron haematoxylin technique. In a few cases, however, when fixation was prolonged to 3 or 4 days and the stain also prolonged to 24 hours in each of the baths of iron alum and haematoxylin, it was possible to detect in a few germinal epithelial cells black bodies bigger in size than mitochondria, recalling the Golgi bodies (Text-fig. 5). On fixing the ovotestis in Nessonov (chrome-osmium-dichromate) and treating with 2 per cent. osmic for 5-7 days in an incubator at 37° C., the Golgi bodies appeared clearly as deep-black curved rods from 6 to 8 in number situated near one pole of the nucleus (Text-fig. 6). Staining the post-osmicated sections with Altmann's acid fuchsin revealed the mitochondrial cloud as a reddish zone around the Golgi bodies, thus proving that the Golgi bodies and the mitochondrial cap are present at one and the same pole of the nucleus.

Differentiation of the Germinal Epithelium

The period of germ-cell differentiation in the desert snail seems to begin by the end of the aestivation period and continues till late spring or early

summer of the following year. At the beginning of this period the germinal epithelium gives rise to the male elements. These elements separate from the wall of the diverticulum and fall into the lumen where they multiply. Almost simultaneously, some of the germinal epithelial cells give rise to nurse cells which gradually grow and spread over the so far undifferentiated germinal epithelial cells. Eventually the wall of the ovotestis diverticulum appears organized into two layers, an inner layer of nurse cells and a peripheral layer of undifferentiated germinal epithelial cells. The cells of the peripheral layer do not differentiate until about the middle of the reproductive cycle, and when they do, they give rise to the female elements.

The earliest symptoms of differentiation in the germinal epithelial cells are the same irrespective of whether male or female elements will ultimately result. In the nucleus, the chromatin blocks which were numerous and small in size in the germinal epithelial nucleus (Text-fig. 4) seem to aggregate together into fewer, more voluminous chromatin masses. Such chromatin masses are no longer completely separate from one another, but are seen to be connected here and there by short, faintly coloured chromatin filaments (Text-fig. 7). Inside the nucleus and a bit to one side one generally finds a nucleolus which takes basic dyes but faintly. Its complete freedom from thymonucleic acid is proved by the negative reaction it gives with Wermel's reagent.

The paranuclear zone of mitochondrial material, already indicated to be present in the cytoplasm of the germinal epithelial cell, becomes in the early germ cell markedly bigger and contains a greater number of big mitochondrial granules (Text-fig. 8).

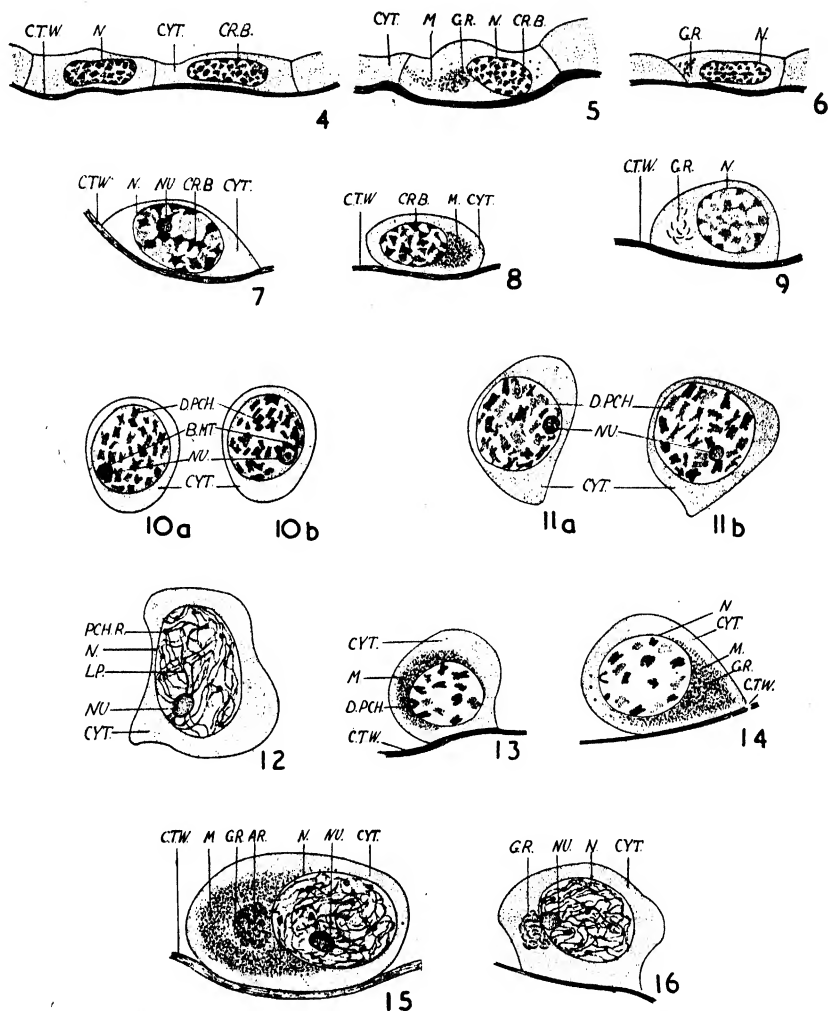
The Golgi bodies of these early germ cells, though still not easily demonstrable by the F.W.A./iron haematoxylin technique, can be brought up marvellously by Nasonov's post-osmication method. As in the case of the germinal epithelium, the Golgi bodies appear as curved osmiophil rods, differing only in being longer and finer. Moreover, these bodies are no longer close to one another but appear dispersed in a bigger zone (Text-fig. 9).

It is from the above-described stage that both male and female lines begin to develop. The name 'Cellule progerminative indifferente', put forward by Ancel (1903) to the germ cell at such a stage, seems most favourable.

In this paper the details of the differentiation of the female elements from the progerminative indifferent cells will be given. The mode of differentiation of the male and nurse cells will be described elsewhere.

The Earliest Female Elements

Nuclear behaviour during the differentiation of the female elements is very characteristic. The chromatin of the progerminative indifferent cell becomes resolved into a few big masses of variable sizes (Text-figs. 10 and 11). Apart from these chromatin masses there exists in the nucleus a voluminous nucleolar formation, which appears constituted of a central sphere out of which extend a few irregular bodies (Text-fig. 10). The central sphere



TEXT-FIGS. 4-16. Differentiation of the germinal epithelial cells into the earliest oocytes.

Figs. 4-6, undifferentiated germinal epithelial cells, Figs. 7-9, progerminative indifferent cells, Figs. 10-16 earliest oocytes. Figs. 4, 7, 10, 11, and 12 from Flemming with acetic, Figs. 5, 8, 13, 14, and 15 from F.W.A., and Figs. 6, 9, 16 from Narsonov's post-osmication preparations. All figs. $\times 1360$.

AR, archoplasm; B.M.T., basophilic material, CR.B, chromatin body; CYT, cytoplasm; D.PCH, double prochromosome; G.R., Golgi rod; LP, leptotene chromosome; M, mitochondria; N, nucleus; NU, nucleolus; PCH.R., prochromosome remnant. Other lettering as before.

consists of a strongly basiphil core and a weakly basiphil rim. The irregular bodies surrounding it are strongly basiphil. Soon afterwards, the irregular bodies disappear and we are left with the central sphere; now staining weakly and homogeneously in basic dyes and forming the earliest oocytic nucleolus (Text-fig. 11).

Focusing the attention now on the chromatin masses (Text-fig. 11), it can be observed that each has the form of a slightly elongated, faintly staining, dual body. Evidence of duality is denoted by the presence of a cleft at both of its extremities. In rare cases that cleft is seen extending along the whole length of the element.

Repeated counts revealed that the number of the dual chromatin masses is 28. The diploid chromosome number of the desert snail, as counted from spermatogonial mitotic plates, is 56. The masses under consideration correspond, therefore, to the haploid number. Being dual, they represent the diploid complement of the female element and, therefore, one is justified at this stage to refer to them as 'double prochromosomes'.

The double prochromosome stage soon gives rise to the unravelling stage. Each double prochromosome becomes resolved into two fine chromatin threads and a spherical remnant. The threads are the early leptotene threads; the remnant on the other hand gives the same colour reactions as the nucleolus and is therefore of the same nature (Text-fig. 12).

While these processes are taking place inside the nucleus, the whole element is increasing in size; the cytoplasm more than the nucleus. The growth period of the female element, therefore, begins the moment it is cytologically detectable. Concurrently, certain important changes take place in the cytoplasmic inclusions.

The mitochondria in the earliest female element constitute a well-defined cap closely applied to one side of the nucleus (Text-fig. 13). This cap appears in F.W.A./iron haematoxylin preparations as a darkly staining cytoplasmic ground matrix containing several jet-black, fine, and slightly coarser mitochondrial granules. Later the mitochondrial cap grows quickly in size and becomes more stainable. Its shape becomes more or less cone like; the point of the cone directed towards the cell periphery, the base closely abutting on to the nuclear membrane (Text-fig. 14). This cone now contains numerous large mitochondrial granules.

In a section passing through a larger oocyte still at the leptotene stage (Text-fig. 15) the mitochondria appear as a huge U-shaped formation, with the end of the U directed towards the nucleus and the base towards the cell periphery. The mitochondrial zone now appears constituted of a tremendous number of well-defined granules which still appear embedded as before in a darkly staining cytoplasmic ground matrix. The latter matrix probably contains mitochondrial material in colloidal dispersion since it does not appear except after mitochondrial techniques.

The Golgi bodies in the youngest oocyte, at the double prochromosome stage, are represented by several slightly curved rods lying nearest to the

nuclear membrane in the zone of the maximal mitochondrial aggregation (Text-fig. 14). About the time the prochromosomes have given rise to the early leptotene filaments, the Golgi bodies appear in F.W.A./iron haematoxylin preparations as curved C-shaped bodies aggregated around a mass of archoplasm (Text-fig. 15). After the Nassonov post-osmication method, the Golgi rods appear as numerous slightly curved rods closely aggregated together; but the archoplasmic mass was not impregnated (Text-fig. 16).

THE GROWTH PERIOD OF THE OOCYTE AND YOLK DEPOSITION

In the previous section the female element was left when it was easily distinguishable as such, namely when its nucleus reached the leptotene stage. During the growth of this early element to the fully formed oocyte, both the nucleus and the cytoplasmic inclusions undergo definite changes. A detailed description of these changes is given below.

The Chromatin and Nucleoli

The juxtaposed chromatin threads of the leptotene nucleus (Text-fig. 12) soon appose side by side and then quickly wind round one another to give the strepsitene double spirals (Text-fig. 17). Very rarely does one find a post-leptotene nucleus with all its pairs of synaptic mates apposed without being relationally coiled. It seems, therefore, that the pachytene stage in the desert snail's oocyte is of very short duration and quickly leads to the strepsitene one. The pachytene, as well as the strepsitene elements of the oocyte, are always disposed more or less radially in the oocytic nucleus, polarized towards a central nucleolus.

In a later oocyte (Text-fig. 18), each strepsitene double spiral shortens, condenses, and becomes more basiphil; now identifiable as a diplotene bivalent. The bivalents are arranged peripherally just underneath the nuclear membrane. Repeated counts from thick sections made it possible to ascertain the haploid number of bivalents, viz. 28.

After diplotene, the oocyte grows a little further and the chromatin begins to enter the diffusion or dispersion, characteristic of a typical germinal vesicle. The bivalents become attenuated and lose almost completely their basiphily (Text-fig. 19). Later the chromatin threads appear as linear series of fine granules (Text-fig. 20). However, until now the identity of the diplotene elements, and sometimes even their duality, is vaguely manifest.

With the progress of growth, the chromatin threads become clumped into a few stellate or irregular formations (Text-fig. 21). Now the identity of the diplotene elements is almost totally masked.

Towards the end of growth, the dispersion of the chromatin reaches its maximum. Now one can see only several chromatin tracts, loose in texture and outline, either free from one another (Text-fig. 22) or partially intercommunicating (Text-fig. 23). Where they are thoroughly free, one can notice that they seem as if suspended in a framework (Text-fig. 22). In the author's

belief, this framework resulted from the coagulation of the enchylema of the germinal vesicle.

As previously indicated, an oocyte at the leptotene stage (Text-fig. 12) possesses a large nucleolus and several small prochromosome remnants which are, in fact, nucleolar in nature.

At the pachytene and strepsitene stages, from two to four nucleoli of fairly large size occur. In Text-fig. 17 three nucleoli are shown; one acting as the polarization centre. Comparing Text-figs. 12 and 17, it becomes evident that some of the material of the nucleoli in the latter oocyte was derived in all probability from the prochromosome remnants.

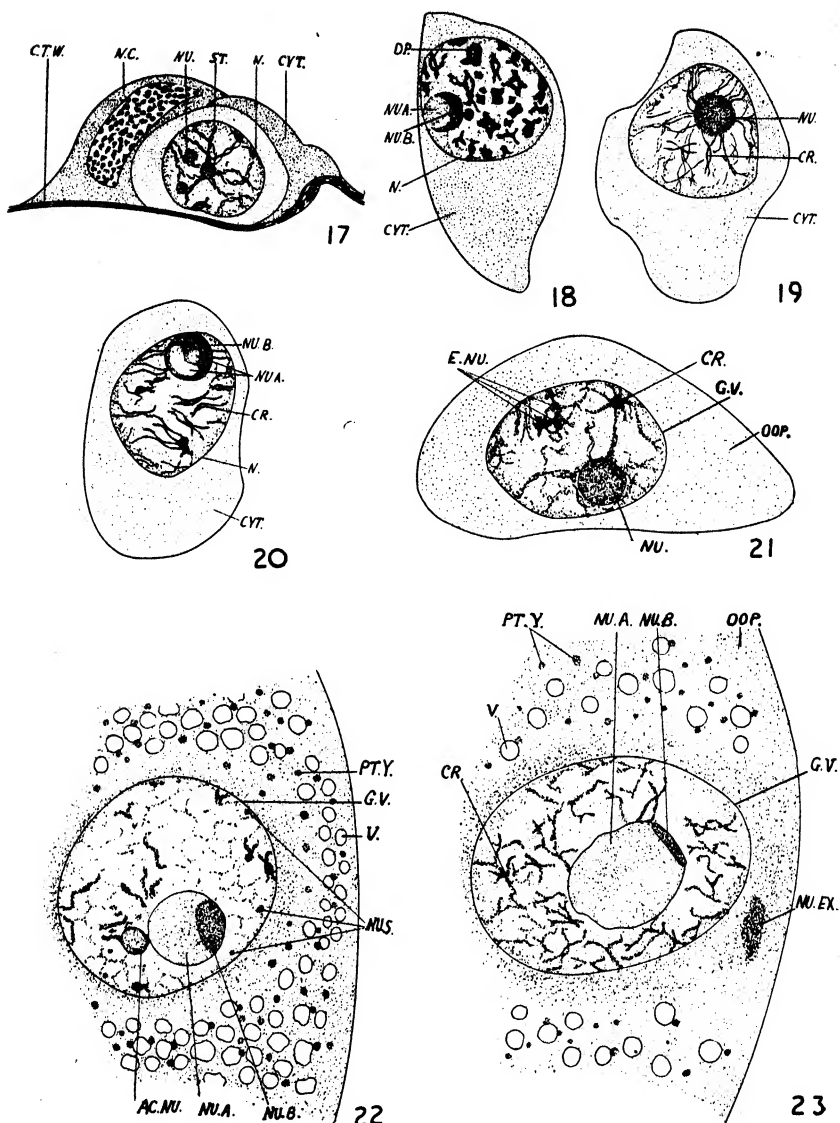
The staining reactions of the nucleoli at the leptotene as well as at the pachytene and the strepsitene stages point to a slight basiphily. However, with Wermel's thymonucleic acid test, the nucleoli do not show the slightest violet tinge. They are, therefore, in spite of their slight basiphil stainability, typical plasmosomes.

During diplotene and early diffusion, nucleoli gradually fuse to give a large single nucleolus. In Text-fig. 18 there occurs the principal nucleolus and a small accessory one. Soon after (Text-figs. 19 and 20), this too fuses with the growing principal nucleolus.

Later in diffusion, when the chromatin of the germinal vesicle appears in the form of stellate formations, there occur, in addition to the large old nucleolus, other very small nucleoli in formation (Text-fig. 21; E.NU). In F.W.A./toluidine blue/eosin preparations the newly developing nucleoli can be identified in the midst of the stellate, now oxyphil (reddish), chromatin clumps, as small spherical bodies which, like the principal nucleolus, stain bluish-red. In the same nucleus one can identify various sizes of such nucleoli ranging from large granules to small spheres. As growth of the oocyte progresses, the newly formed nucleoli gradually fuse with the old principal nucleolus. In the germinal vesicle of the oocyte depicted in Text-fig. 22 there occurs, apart from the principal nucleolus, a much smaller accessory one, closely associated with it. This, too, is destined to fuse with the principal nucleolus, so that towards the end of the growth period we are left with a single giant nucleolus towards the middle of the germinal vesicle (Text-fig. 23).

The moment the oocyte's principal nucleolus reaches a fairly large size, at or shortly before chromatin diffusion, its stainability changes. Originally taking weakly and homogeneously the basic dyes, it now stains heterogeneously; certain parts stain more acidophil, others more basiphil. In Text-fig. 18 the acidophil part (NU.A) is rounded up excentrically in the nucleolus, and the basiphil part (NU.B) constitutes a peripheral crescentic zone. In Text-fig. 20 there can be seen in the midst of the nucleolus two adjacent acidophil zones, one spherical, the other bean-shaped; the rest of the nucleolus is basiphil.

In a fairly grown oocyte (Text-fig. 22), the major part of the nucleolus appears acidophil, the basiphil material being contained in a small zone to one side. Also, just underneath the nuclear membrane a few small nucleolar



TEXT-FIGS. 17-23. Behaviour of the chromosomes and the nucleoli in the growing oocytes.

Fig. 17, strepsitene stage; Fig. 18, diplotene stage; Figs. 19-23, successive stages in the denucleination of the chromosomes during the formations of the oocytic germinal vesicle. All figures are from Flemming with acetic/Scott preparations. Figs. 17-21 $\times 1360$; Figs. 22 and 23 $\times 680$.

AC.NU., Accessory nucleolus; CR, chromatin; E.NU., early nucleoli; G.V., germinal vesicle; N.M., nuclear membrane; NU.A., acidophil part of nucleolus; NU.B., basiphil part of nucleolus; NU.EX., nucleolar extrusions; NU.S., nucleolar spherules; OOP., ooplasm; P.NU., principal nucleolus; PT.Y., proteid yolk; ST, strepsitene bivalent; v, vacuole. Other lettering as before.

spherules staining basiphil can be detected (NU.S). Soon afterwards the nucleolar basiphil material markedly decreases; now the whole nucleolus is acidophil, except for a very small basiphil part extending to one side (Text-fig. 23), NU.B.). Also the small basiphil nucleolar spherules are no longer apparent. At the same time, a mass of material, staining just like the basiphil part of the nucleolus, appears outside the nuclear membrane in the ooplasm.

Although no granular emission has been observed either from the nucleolus into the nucleoplasm, or from the nucleus into the cytoplasm, yet the coincidence of the appearance of the ooplasmic mass with the decrease in the basiphil nucleolar material seems to furnish inferential evidence for some type of nucleolar extrusion. The extrusion, most likely, is of material in solution, as was tentatively suggested by Harvey for *Ciona* (1927), *Carcinus* (1929), and *Antedon* and *Asterias* (1931). The probability is that the nucleolar basiphil material transformed into a liquid phase or rendered in solution, passes as such through the nuclear membrane and once in the ooplasm recollects again to construct formed bodies. The small nucleolar spherules, observed just to the interior of the membrane of the germinal vesicle in some oocytes, may have resulted from the coagulation of the emitted nucleolar material before its final exit into the ooplasm.

Similar ooplasmic basiphil masses were observed and described by Gatenby (1917) in the oocytes of the *Helix aspersa*. However, he did not discuss either their origin or their nature.

The Cytoplasmic Inclusions and Deutoplasmic Bodies

In the early oocytes we have already seen that the mitochondrial zone consisted of a number of granular mitochondria embedded in a darkly staining ground matrix. At about the leptotene stage the same zone appeared as a fairly large U-shaped formation.

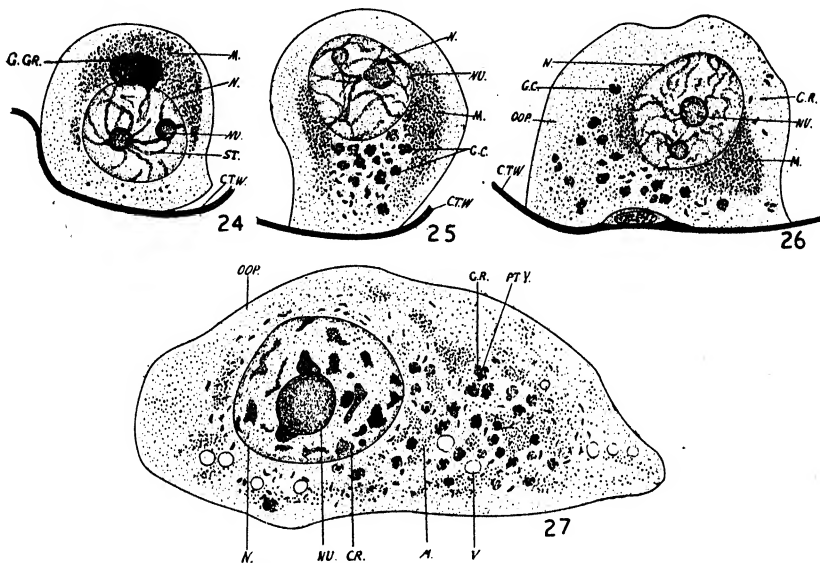
Later, at the pachy-strepsitene stage (Text-fig. 24), the mitochondrial zone increases still more in size and now extends from the nuclear membrane to very near to the cell periphery. The mitochondrial granules themselves are larger in size and darker in stainability than before. Away from the mitochondrial zone a few separate mitochondrial granules may appear around the nucleus. Soon after (Text-fig. 25), the mitochondrial zone seems to be divided into two subequal groups.

As the oocytic growth becomes pronounced, the mitochondrial granules quickly increase in number and begin to disperse in the ooplasm. Text-fig. 26 represents an early stage in mitochondrial dispersion. Here again two mitochondrial groups are detectable; in one group the close aggregation of the mitochondrial granules is still preserved, in the second, mitochondria have partially dispersed. The mitochondrial granules in the dispersion zone are slightly finer than those in the other zone where dispersion is not pronounced.

As the oocyte reaches a fairly good size (Text-fig. 27), just at the beginning of yolk deposition, mitochondria become dispersed somewhat unevenly in the

cytoplasm. Groups of fine mitochondrial granules are met with here and there in the oocyte's cytoplasm.

Towards the end of growth (Text-figs. 33 and 34), when yolk deposition has well progressed, mitochondria appear evenly distributed all over the ooplasm. Now most of the mitochondrial granules are undoubtedly larger than those in the earlier oocytes of the stage shown in Text-fig. 27, but they are still much smaller than the smallest yolk spheres.



TEXT-FIGS. 24-27. Mitochondria, Golgi bodies, and yolk in oocytes at the early stages of their growth.

All figs. from F.W.A. preparations; $\times 1360$.

G.C., Golgi complex; G.G.R., localized Golgi group. Other lettering as before.

It was previously indicated that the oocyte's Golgi elements at the leptotene stage appeared as a group of osmiophil slightly curved rods (Text-figs. 15 and 16). At the stages immediately following (pachytene and strepsitene), the number of the Golgi rods appears to be greater and their size larger (Text-figs. 24 and 28). Still they lie aggregated in a group to one side of the nucleus.

As the oocyte grows, the Golgi group also enlarges, both by enlargement of the individual rods and by the appearance of more smaller rods presumably through fragmentation of the older rods. By the stage depicted in Text-fig. 29, the Golgi juxtannuclear group appears large, and also several rods appear to have migrated into the cytoplasm from the main group.

In slightly older oocytes (Text-fig. 30) the Golgi elements appear widely distributed almost all over the ooplasm. Some of the scattered Golgi rods lie singly in the cytoplasm, but others are grouped to form complexes very

characteristic of the oocyte of the desert snail at, and immediately after, the stage at issue (Text-figs. 30 and 31).

In F.W.A./iron haematoxylin preparations, the Golgi bodies were usually detected (Text-figs. 24, 25, 26, 27). The appearance of the oocytic Golgi complex after this technique is typical. Each complex consists of an archoplasmic mass with the Golgi rods arranged on its perimeter. The curved Golgi rods are disposed with their concave sides facing centrally, i.e. towards the archoplasm.

After the scattering of the Golgi bodies has fairly proceeded, yolk deposition sets in. From the beginning, this deposition takes place in close association with the Golgi bodies. In the interior of the Golgi complexes, or on the concave sides of the separate Golgi rods, there appear minute yolk spherules (Text-figs. 27 and 31). While still in association with the Golgi rods the yolk granules seem to enlarge, because within different Golgi complexes granules of various sizes were seen (Text-fig. 31).

At this early period of yolk deposition, the ooplasmic masses referred to before are very prominent and occupy a juxtanuclear position (Text-fig. 31, NU.EX).

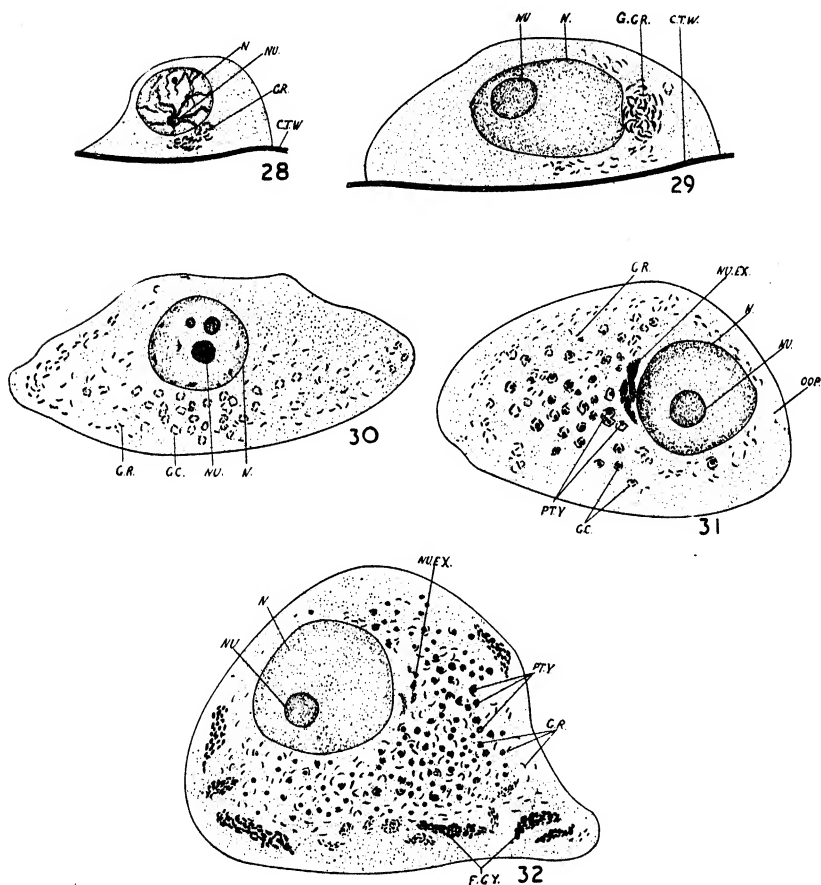
As vitellogenesis proceeds still farther, the cytoplasmic inclusions of the oocyte appear as shown in Text-fig. 32. Most of the Golgi complexes have now separated into their constituent rods. Rarely, some rods still appear associated in twos to form V-shaped formations. While some yolk spherules still appear associated with the Golgi bodies, others have migrated off into the ooplasm. Every step in this migration can be traced. The yolk spherule is first severed from the Golgi rod, then appears a short distance from it, and at last it is completely separate.

The ooplasmic masses are still manifest at this stage, but are undoubtedly much smaller than before (compare Text-fig. 31 with Text-fig. 32). Later, these masses are thoroughly lost to view, their material probably contributing to the raw material used in yolk formation.

After the Golgi bodies are severed from the yolk granules, they soon run together and form clumps of variable sizes scattered here and there in the ooplasm, especially towards the periphery (Text-figs. 32 and 33). In the early phases of the process, the rods of the clumps do not differ much from the Golgi rods of previous stages, except for a slight increase in osmiophily (the small clumps in Text-fig. 32). Later the rods increase in girth and thus appear almost oval (Text-fig. 33). Also they become much easier to impregnate with osmium, appearing black in chrome-osmium material even without post-osmication. Further, applying Sudan III and Scharlach R on fresh and formalin-fixed material, it was found that the oval elements of the clumps gave a positive test; these elements, therefore, must contain fat.

Most helpful and clarifying is the following test: post-osmicated sections were mounted in turpentine, a cover slipped over, and the extraction of osmium from the elements of the clumps was observed under the microscope. Gradually the girth of the elements decreased, till after a lapse of half an

hour their girth fell to its state before the clumping stage, being now of just the same girth as the unchanged Golgi rods of the early oocytes in the same section. Up till the end of the first hour no further appreciable extraction was



TEXT-FIGS. 28-32. The Golgi bodies before and during vitellogenesis in the growing oocytes.

All figs. from Nassonov post-osmication preparations. Figs. 28-30, $\times 1360$; Figs. 31 and 32, $\times 680$.

F.G.Y, fatty Golgi yolk. Other lettering as before.

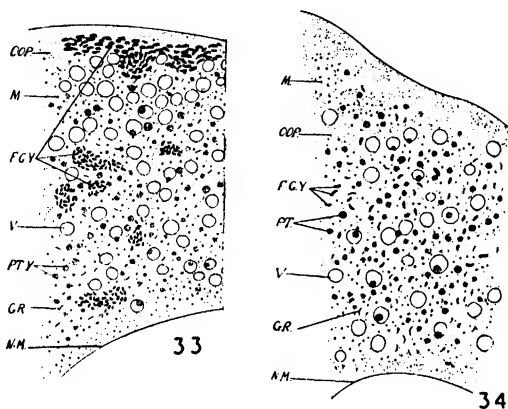
noticed. The slide was then uncovered and transferred to a jar of turpentine. Even after 3 hours neither the residual rods of the clumps in grown oocytes, nor those of the young oocytes, were decolorized.

On rare occasions, it must be noted, the Golgi bodies did not clump together in groups, but remained widely scattered in the ooplasm (Text-fig. 34).

However, in these cases also the increase in the girth of rods due to their loading with fat was plainly evident.

The natural conclusion, therefore, is that in the late growth period of the oocyte, some of the Golgi rods become loaded with a certain fat. The original Golgi element in the composite body, however, always preserves its identity and does not transform itself into fat.

The cytoplasm of the full-grown oocyte of the desert snail contains a fairly large number of vacuoles. These vacuoles appear in abundance fairly late in



TEXT-FIGS. 33 and 34. Portions of the ooplasm towards the end of growth.
From Nassonov/Altmann's fuchsin/aurantia preparations; $\times 1020$, lettering as before.

the oocytic growth, though a few may appear in earlier stages. In the fairly young oocyte depicted in Text-fig. 27, a few vacuoles were seen. The content of these vacuoles is most probably watery, since it gives negative reactions with all the fixing reagents used.

Between the vacuoles of the oocyte are embedded the four elements: yolk spheres, mitochondria, and the normal and fat-loaded Golgi bodies (Text-figs. 33 and 34). In some instances, yolk spheres are seen in the interior of the vacuoles. This may be attributed to the mechanical dislocations during preparation, since in early oocytes (Text-fig. 27) the first-appearing vacuoles did not envelop yolk spheres.

Even fairly late in growth, the distinction between the different cytoplasmic inclusions of the oocyte of the desert snail is by no means a difficult matter. In F.W.A./iron haematoxylin/erythrosin preparations the fat-loaded Golgi bodies appear as black oval grains; the normal Golgi bodies as fine curved blackish rodlets; the mitochondria as black granules of various sizes; the yolk spheres take the plasma stain faintly and thus appear yellowish-red. Most helpful, also, is the Nassonov/Altmann combination technique. After this method the mitochondria stain deep red, the fat-loaded Golgi bodies appear

as jet-black grains, the normal Golgi bodies as fine curved black rodlets, and the yolk spheres as yellowish-brown spheres.

On the application of Baker's acid-haematein technique it was possible to detect in the cytoplasm numerous small blue-black granules corresponding to the mitochondria, a few slim dark-blue rodlets probably corresponding to the unchanged Golgi bodies, many large pale bluish-brown spheres corresponding to the proteid yolk spheres, and lastly yellowish spheres that correspond to the fat-loaded Golgi bodies.

When the acid-haematin test is applied after pyridine extraction, only the nucleoli inside the nucleus and the proteid yolk spheres in the ooplasm gave positive reaction, the nucleolus being stained a much deeper blue than the yolk spheres.

The acid-haematin/pyridine extraction combination, therefore, shows that lipin is not present in any appreciable concentration in the desert snail's oocytes except in the mitochondria and the unchanged Golgi bodies.

The Centrifuged Oocyte

In the fully grown oocyte, the different ooplasmic elements are more or less haphazardly mixed. On centrifuging, however, it was found that these elements separated in successive strata. Naturally this gives a better chance for the determination of the physical and histochemical characteristics of the different elements in each stratum.

The ovotestis was immersed in an isotonic Ringer's solution or snail's blood, and then centrifuged at a speed of 3,500 revolutions per minute for half an hour. Immediately after centrifuging, the serum or Ringer was poured off, and the fixatives instantaneously applied. The centrifuged material was treated with mitochondrial and Golgi techniques and with Baker's formol-calcium/acid-haematein method.

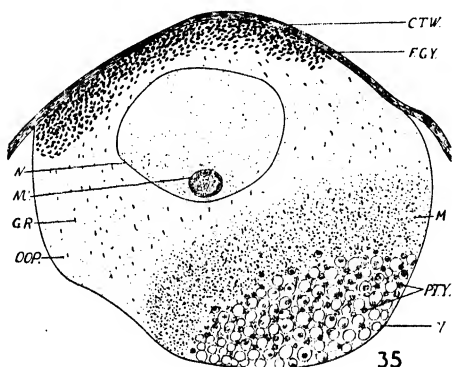
The cytoplasmic bodies of the centrifuged oocyte (Text-fig. 35) appear stratified in four layers. The uppermost layer occupies 10 per cent. of the volume of the oocyte, and contains oval, fairly large bodies. These bodies reduce osmic acid easily, appearing jet-black after post-osmication and even in unstained chrome-osmium sections. In formalin-fixed material, subsequently stained in Sudan III or Scharlach R, these bodies stained brilliant red. There is little doubt, therefore, that this layer represents the fat-loaded Golgi elements (Text-fig. 35, F.G.Y.).

Just underneath the above-mentioned layer there appears a layer of clear cytoplasm occupying about 50 per cent. of the volume of the oocyte and containing several unevenly scattered Golgi rods (Text-fig. 35, OOP). In this layer the nucleus lies with the nucleolus shifted towards the centrifugal pole. Sometimes the nucleolus shattered the nuclear membrane and became thrown off for a short distance into the underlying cytoplasm.

The third layer appears as a band of granules extending underneath the clear area. These granules appear yellowish in unstained chrome-osmium and Mann-Kopsch preparations, but stain strongly in Altmann's acid fuchsin,

crystal violet, iron haematoxylin, and Baker's acid haematein. This layer is, therefore, undoubtedly mitochondrial (Text-fig. 35, M).

The heaviest layer at the centrifugal pole of the oocyte shows a collection of vacuoles and numerous coarse and fine spherules. The contents of the vacuoles give negative tests for fats, proteins, and glycogen. It seems, therefore, that their contents are essentially watery. The spherules appear yellowish-brown in unstained Nassonov and Mann-Kopsch preparations, and stain weakly or not at all in Altmann's acid fuchsin and haematoxylin and



TEXT-FIG. 35. The centrifuged oocyte.

From Nassonov/Altmann's fuchsin/aurantia preparations. $\times 340$. Lettering as before.

Baker's haematein. There is little doubt, therefore, that these represent the true yolk spheres (Text-fig. 35, PT.Y).

In the mitochondrial and yolk strata one may occasionally find a few Golgi rods. These seem to have been entangled with the granular elements (mitochondria and yolk spheres) in their centrifugal drift.

Brambell (1924), after centrifuging the oocytes of *Helix aspersa*, found a single layer of swollen mitochondria at the centrifugal pole. These he took to represent what he called 'mitochondrial yolk'.

The centrifuged oocyte of the desert snail shows towards the centrifugal pole two layers and not one: an upper layer of mitochondrial granules of various sizes and a lower one of true yolk spheres. The latter, as previously shown, have nothing to do with the mitochondria, being formed under the influence of the Golgi bodies.

DISCUSSION

During the last 25 years, cytological literature has been full of valuable publications seeking to disclose the mechanism of yolk formation in growing oocytes. At present there are two competing schools of thought.

According to one school (Nath and co-workers, 1924 et seq.), fat is formed in relation to the Golgi bodies, whereas yolk is derived from nucleolar extrusions

or arises *per se* in the ground cytoplasm. This method of deutoplasmogenesis was described in Crustaceans (*Palaemon*—Bhatia and Nath, 1931, and *Paratelson*—Nath, 1934); Chilopods (*Lithobius*—Nath, 1924, and *Otostigmus*—Nath and Husain, 1928); Spiders (*Crossopriza*—Nath, 1928, and *Plexippus*—Nath, 1934); Insects (*Luciola*—Nath and Mehta, 1929, *Periplaneta*—Nath and Piare Mohan, 1929, and *Culex*—Nath, 1929); Fishes (*Rita* and *Ophiocephalus*—Nath and Nagia, 1931, and also Nath, 1934); Amphibia (*Rana*—Nath, 1931 and 1934); Reptiles (*Emyda*—Nath and Azez Ahmed; quoted in Nath, 1934) and Birds (*Gallus*—Nath, 1934).

The Golgi bodies, according to these authors, are in the shape of vesicles, with osmiophil rims and osmiophobe cores. At some stages in oogenesis fat is deposited within the Golgi vesicles, thus causing them to swell into the fatty yolk spheres (see especially Nath, 1930).

Applying Scharlach R and Sudan III on fresh and formalin-fixed oocytes, Nath (1934) could, in several instances, stain his 'Golgi spherules' red in the older but not in the younger oocytes. This he considered as further evidence in favour of the Golgi origin of fatty yolk.

Of the upholders of the principle of origination of fat in connexion with Golgi bodies, apart from Nath's school, may be cited King (1926), working on *Oniscus*; Gresson (1929, 1931, and 1933), working on three species of Tenthredinidae and also *Periplaneta orientalis* and *Stenophylax stallatus*, and also Bell (1929), who showed that even in the male germ cells fat may be derived from the Golgi bodies.

On the other hand, a group of competent observers have maintained that the Golgi bodies are somehow concerned in the production of true (proteid) yolk.

Wheeler (1924) working on *Pleuronectus*, Weiner (1925) on *Lithobius* and *Tegeneria*, and Steopoe (1926) on *Nepta* have all found that yolk is formed in the periphery of the oocyte among and in intimate relation to the Golgi bodies. Also Gardiner (1927), working on *Limulus*, found that yolk appears in regions of the ooplasm where Golgi bodies and mitochondria are maximally aggregated. He suggested that yolk arose through the interaction of mitochondria, Golgi bodies, and nucleolar extrusions. To Harvey, L. A. (1929 and 1931), the credit of establishing the role of the Golgi bodies in the production of true yolk must be attributed. In his works on *Carcinus* and also *Antedon* and *Asterias*, he maintains that the raw material of this yolk is largely derived from the exterior of the oocyte, but it is also partially provided by the nucleolar extrusions. From this raw material yolk is synthesized, presumably through the activity of mitochondria, and then becomes condensed under the influence of the scale-like Golgi bodies into droplet form, the process being described as 'physical condensation rather than chemical synthesis'. As to fat, Harvey believes that it arises *per se* in the ground cytoplasm.

In the present work on the desert snail, it was found that true yolk (largely proteid) originates under the influence of the Golgi elements. Frequently the primordium of the yolk sphere appears in the interior of a Golgi circle,

formed by the grouping of some Golgi rods end to end. This positional relationship between the early yolk spheres and the Golgi rods indicates, as Harvey (1929) notes for similar conditions in *Carcinus*, 'that the Golgi elements are mostly concerned with the final stages of condensation of yolk in the cytoplasm, whatever may be their relations in yolk synthesis' (loc. cit., p. 168).

The claims of Brambell (1924) that some of the mitochondrial elements of the oocytes of *Helix* and *Patella* swell into 'mitochondrial yolk' have nothing to uphold them from the present study. Late in growth, mitochondrial granules grew almost imperceptibly in size, but still remained very much smaller than the smallest yolk spherules. Further, all through vitellogenesis the mitochondrial granules preserved the histochemical characteristics of mitochondria and reacted to stains and fixatives differently from yolk. Harvey (1929) favoured the possibility that the role of mitochondria in vitellogenesis is not the final condensation of the yolk spherules, but rather the synthesis of the definitive yolk molecules from the raw material prevailing in the ooplasm. This is probably the case in the desert snail as well, since no positional relationship occurred between the mitochondrial elements and the developing yolk spherules.

Nucleolar extrusions were described in Mollusc oocytes only by Ludford (1921b). However, Gatenby (1917), and even earlier Ancel (1903) detected in the cytoplasm of the oocytes of Helicids certain masses which disappeared late in growth. The present work on the desert snail revealed that similar masses are in all probability nucleolar extrusions. These subsequently disappear, their material probably providing at least some of the raw material used in yolk synthesis.

Fat, in the oocytes of the desert snail, deposits on the Golgi rods, after the latter have left the yolk spheres and clumped in groups especially towards the periphery of the ooplasm. Brambell's (1924) claim that fat (his Golgi yolk) in the oocytes of *Helix aspersa* arises through direct metamorphosis of the Golgi rods is not substantiated by the present work. Careful histochemical tests (pp. 173-5) showed that the definitive Golgi rod always preserved its identity and only became loaded with a variable amount of free fat.

The course of vitellogenesis in the desert snail serves marvellously in the settlement of the long-lived controversy between L. A. Harvey and Nath and co-workers as to the role of the Golgi bodies during vitellogenesis. In agreement with Harvey's view, true yolk in the desert snail's oocyte arises under the influence of the Golgi rods. After these rods are released from this function, they become loaded with fat, a fact in harmony with the essence of the view put forward and defended by Nath's school.

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SUMMARY

1. The histological structure of the ovotestis is briefly described.
2. The germinal epithelium gives rise to both categories of germ cells (male and female) as well as to the nurse cells.
3. During the differentiation of the germinal epithelial cell towards the germ line, it passes through a certain stage, 'the progerminative indifferent stage', before it is polarized either towards the male or female line.
4. A detailed description of the mode of differentiation of the progerminative indifferent cell into the earliest female element is given.
5. Mitotic multiplication of the early female elements never occurs. Nevertheless the diploid chromosome complement is represented by pro-chromosomes.
6. During the oocytic meiotic prophases, parasynaptic conjugation of the chromosomes is quickly followed by relational coiling of the synaptic mates and the formation of the strepsitene double spirals.
7. The strepsitene double spirals give rise to the diplotene bivalents of the haploid count 28.
8. The steps of chromatin diffusion that lead to the construction of the typical oocytic germinal vesicle are described.
9. Changes in size, number, and stainability of nucleoli during oocytic growth are recorded. Extrusion of basiphil material from the nucleolus into the ooplasm is highly probable.
10. The mitochondrial granules at the beginning of the period of active oocytic growth form a huge zone to one side of the nucleus. Later they increase in number and widely scatter in the ooplasm. It is held (in favour of Harvey's view, 1929 and 1931) that mitochondria are probably concerned in the chemical synthesis of yolk from raw material provided in the ooplasm.
11. The Golgi bodies in oocytes are in the form of rods and not vesicular. In the early oocyte they form a group to one side of the nucleus. Eventually they disperse in the ooplasm either singly or in small typical complexes. Each of the latter is constituted of a few rods arranged end to end as if on the perimeter of an irregular circle.
12. The earliest yolk spheres (largely proteid) appear in the interior of the Golgi complexes or on the concave sides of the separate Golgi rods.
13. The Golgi rods become severed from the yolk spheres and migrate mainly towards the periphery of the oocyte where they collect in clumps.
14. The elements of the Golgi clumps become loaded with an unsaturated free fat. The original Golgi rod always preserves its identity and never transforms itself into fat.

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The Distribution of Alkaline Phosphatase in the Skull of the Developing Trout

BY

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(With two Plates)

INTRODUCTION

THIS work is part of a comparative study of the histological and cytological distribution of alkaline phosphatase in developing teleost and elasmobranch fishes, made with a view to throwing some light on the mechanisms involved in calcification and ossification. Whereas the adult mammalian skeleton is almost completely ossified, in the lower vertebrates cartilage persists side by side with bone. In elasmobranchs bone does not occur but calcium deposits are formed in the cartilage. Here calcification can be studied independently from ossification.

No histochemical study of phosphatase in lower vertebrates has so far been published. There is, however, evidence that phosphatase exists in teleosts as well as elasmobranchs, and is closely similar to the alkaline phosphatase found in ossifying parts of mammals (Bodansky, Bakwin, and Bakwin, 1931; Roche and Bullinger, 1939). The latter authors found a correlation between the degree of ossification and the phosphatase content in different species of fish and at different stages of development in the same species. A study of phosphatase in relation to the growth of scales in elasmobranchs and teleosts leads Roche, Collet, and Mourgue (1940) to conclude that here, as in the higher vertebrates, phosphatase is concerned with rapid osteogenesis.

These results suggest that the same biological mechanism is operative in the calcification of the skeleton in fish as in mammals. In view of this a systematic study of the distribution of phosphatase and calcium salts in developing teleost and elasmobranch embryos seemed of interest. This incidentally provides material for the study of phosphatase in the embryo generally. Studies so far undertaken in this direction were confined to young fowl embryos (Moog, 1944) and the heads of rat embryos (Horowitz, 1942).

This paper deals only with ossification in a typical teleost. The results obtained with elasmobranch embryos will be published separately. The trout (*Salmo* spp.) was chosen because it can conveniently be reared in the laboratory. Moreover the development of *Salmo* has been intensively studied from

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the morphological point of view (Schleip, 1903; Gaupp, 1906; Böker, 1913; Saunderson, 1935; de Beer, 1927 and 1937). Emphasis is here placed on the phosphatase content and degree of calcification rather than on the anatomical aspects of bone formation.

In the trout as in all teleosts, bones arise in a variety of ways. They may on the whole be classified into membrane bones, cartilage bones, and mixed ossifications.

I am here mainly concerned with the early stages of bone formation, i.e. the formation of the pre-osseous matrix—either within the mesenchyme as in membrane bone formation, or below the perichondrium as in cartilage bone formation—and the subsequent calcification of the matrix with its accompanying variations in phosphatase content and distribution. Examples of the different types of ossification will be described in detail. There was complete agreement of the phosphatase distribution and general appearance among fish at the same stage of development (not necessarily of the same age, but usually of the same length). The structures described were therefore chosen from whichever specimens showed the particular features best. The nomenclature used is that of de Beer (1937).

The following cartilages are suitable for the study of phosphatase in relation to their development and ossification:

Neurocranium. The anterior wall of the auditory capsule and the lateral commissure give rise to the pro-otic bone which later involves the basal plate and the anterior end of the parachordals. The basi-occipital appears in the form of perichondral lamellae of the hind portion of the basal plate on each side of the notochord.

Splanchnocranium. Meckel's cartilage and the autodontary; the pterygo-quadrate giving rise to the autopalatine, the metapterygoid, and the quadrate; the hyosymplectic cartilage giving rise to the hyomandibula and the symplectic; other visceral cartilages.

The following structures illustrate *membrane bone* development: (i) the parasphenoid, a typical flat membrane bone which develops in the mesenchyme between the trabecula and the mucous membrane of the mouth. (ii) The dermodontary, i.e. the membrane bone portion of the dentary which is a mixed ossification. Since it develops in close relationship to Meckel's cartilage the structures composing the lower jaw will be described together after Stage 1. (iii) The maxilla, a membrane bone of the upper jaw entering into relationship with teeth. The premaxilla develops on similar lines. (iv) The pre-opercular which lodges part of the mandibular lateral line canal and lies postero-lateral to the symplectic cartilage.

Details of the above structures are described in my thesis (Lorch, 1948). Here only examples of the different types and stages of ossification are given.

The terms 'positive', 'strongly staining', 'black', or 'grey' refer to the presence of phosphatase. No attempt has been made to give a strictly quantitative estimate of the phosphatase content of the tissues. The degree of staining after various incubation times is the sole criterion for stating that a tissue is

'strongly positive' or 'contains little phosphatase'. A 'negative' Gomori reaction does not necessarily imply complete absence of the enzyme since there is a considerable loss during the preparation of the tissues.

MATERIAL

Specimens of brown and rainbow trout were reared from 'eyed' ova in the laboratory. It was decided to use length rather than time from hatching as an indicator of development. About 40 specimens ranging from 10 to 40 mm. were examined.

It was found convenient to divide the developing trout into groups of approximately the same length as follows:

<i>Stage (for reference)</i>	<i>Length (mm.)</i>	<i>No. of specimens examined</i>
1	10-12	6
2	15.5-17	5
3	20	6
4	21-23	11
5	29-38	10

METHODS

Samples of trout were removed at intervals after hatching. Any yolk present was dissected off and the length of the specimen measured to the nearest millimetre. Younger alevins were anaesthetized in 10 per cent. alcohol before fixation to prevent curling and to facilitate measurements. Specimens intended for visualization of phosphatase were fixed in 80 per cent. ethyl alcohol. Some specimens of each stage were fixed in Bouin's fluid for general histology and some in 5 per cent. neutral formalin for the preparation of whole mounts of the skeleton stained with alizarin red S by the method of Hollister (1934). The latter helped in identifying the calcified structures and gave an indication of the areas in which calcification first occurred.

Most of the specimens intended for the phosphatase technique were cut undecalcified. Some of the older specimens were decalcified using citrate buffer for 2 to 5 hours according to my method (Lorch, 1947). There was a slight reduction in the phosphatase content. Groups of serial sections (8-10 μ) were mounted on alternate slides, one being incubated and the other used as control. The slides were taken through celloidin to distilled water. Phosphatase was visualized by the method of Gomori (1939) and Takamatsu (1939). Minor changes in the composition of the substrate were made as follows:

2 per cent. calcium nitrate 10 ml., 2 per cent. magnesium chloride 10 ml.,
4 per cent. sodium β -glycerophosphate 10 ml., 1 per cent. sodium barbitone 70 ml.

Slides were incubated 1–18 hours at 28° C. and pH 9.4. Subsequent treatment was as described by Danielli (1946). Some sections were counterstained with dilute eosin.

Where the amount of preformed phosphate was considerable (specimens over 20 mm.) it became desirable to show the calcium salts and phosphatase in different colours, hence the gallamine blue technique was applied. The reasons for choosing this method as well as its limitations are discussed in my paper on mammalian bones (Lorch, 1947).

The following histological stains were used: Heidenhain's 'Azan', van Gieson's connective tissue stain, Ehrlich's haematoxylin and eosin, and von Kossa's silver nitrate method for bone salts.

RESULTS

Stage I (10–12 mm.)

A. General Distribution of Phosphatase

In all tissues which contain phosphatase the reaction is most marked in the nuclear membranes and nucleoli. The cytoplasm on the whole is negative, but it is not always easily recognizable since the degree of shrinkage and distortion due to alcohol fixation is considerable in the younger embryos. The mesenchyme stains irregularly—nearly all areas show some degree of activity—and the most marked concentration of positive nuclei occurs at the angles of the mouth and in the vicinity of the jaw cartilages. The central nervous system displays positive nuclei and fibres; the intensity of the reaction is variable. The fibrous membranes of the brain are strongly positive. The cells of the retina display slightly positive nuclei at some levels. Nuclei of striated muscle, and of the endothelial cells lining blood-vessels, are positive. The epithelium is negative.

B. Skeletal Tissues

All the cartilages of the chondrocranium display nuclear phosphatase, more or less marked, and in some places the matrix is also slightly positive, more so in the 10-mm. than in the 12-mm. specimens.

Neurocranium. Anterior to the region of the articulation of the lower jaw only the chondrocyte nuclei in the trabeculae are positive, but posteriorly, i.e. near the parachordals, the matrix too displays slight phosphatase activity. The cartilage cells are large and the area occupied by matrix relatively very small. The nuclei have a granular appearance. The cytoplasm is very faintly positive, and can just be observed in slides incubated 15 hours. The perichondral fibroblasts are strongly positive and fine black fibrils are seen in the surrounding mesenchyme which stains most strongly dorsally to the trabecula (Pl. 1, fig. 1). The parachordals contain strongly positive nuclei. The matrix stains positive in well-defined zones where the cartilage is cut near its surface. In the auditory capsules only the nuclei are positive. The perichondrium is still strongly positive on the dorsal (brain) side. More distally the para-

chordals lose their extracellular phosphatase except in the zone adjacent to the notochord: the nuclei remain positive throughout.

The notochord sheath consists of 3 layers: an inner layer of cubical cells with strongly staining nuclei, a middle layer, seemingly structureless and free from phosphatase, and an outer layer of elongated fibroblasts closely packed and strongly positive. The substance of the notochord itself is free from phosphatase.

Splanchnocranium. Meckel's cartilage is completely negative in its anterior tip where the pair of cartilages join. The middle and posterior regions contain nuclear phosphatase, the enzyme being most concentrated in the nucleoli and nuclear membranes. There is no extracellular phosphatase in Meckel's cartilage. The distribution of phosphatase in the mesenchymal nuclei of the lower jaw is interesting: they are strongly positive lateral to, moderately so ventral to, and negative dorsal to Meckel's cartilage. This is shown in Text-fig. 1, and will be further discussed under membrane bone development.

Pl. 1, fig. 2, shows the quadrate process and the hyosymplectic cartilage of a 12-mm. specimen. The mesenchymal nuclei are strongly positive. The hyosymplectic also shows nuclear phosphatase and some extracellular phosphatase at the periphery. The basihyal displays positive nuclei only after long incubation times, while the other branchial cartilages show strongly staining chondrocytes and perichondral fibroblasts after 6 hours' incubation. There is phosphatase in the striated muscle nuclei.

The first stages in the formation of *membrane bone* are illustrated by the dermodentary. This is seen as a minute fragment of uncalcified osteoid at some levels only. It forms a thin lamella central and lateral to Meckel's cartilage, i.e. in the region where the mesenchymal nuclei are most strongly positive. The osteoid contains phosphatase (Text-fig. 1).

The pre-opercular resembles the dermodentary. The maxilla is present as a very small incompletely calcified rod angular in cross-section. There is as yet no sign of the parasphenoid.

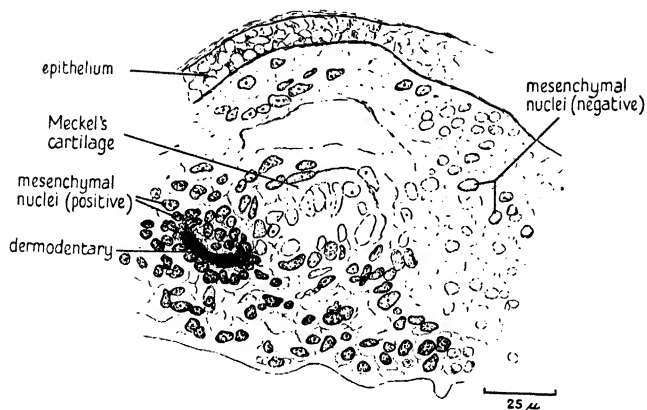
Summary of Stage 1

Nuclear phosphatase is widespread in skeletal as well as non-skeletal tissues. There are occasional traces of enzyme in the cartilage matrix. Osteoid is present in some phosphatase-rich areas of mesenchyme. There is no calcification.

Stage 2 (15.5-17 mm.)

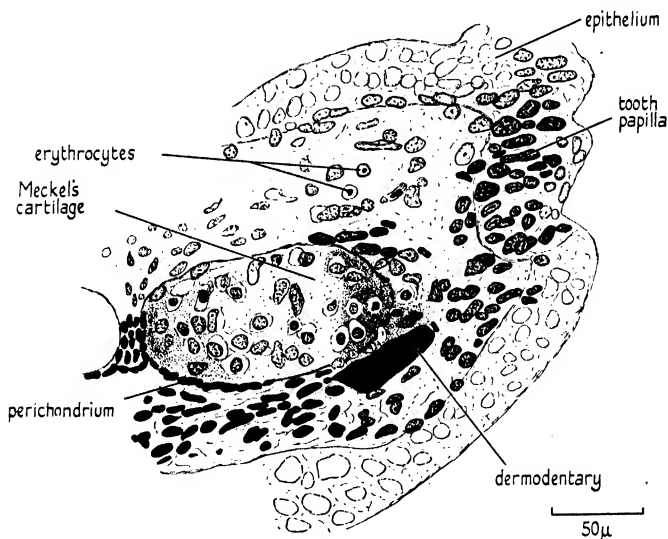
A. General Distribution of Phosphatase

This does not differ greatly from the distribution at the previous stage. But a new zone of strongly positive mesenchyme is seen just below the epithelium of the lower jaw anterior to the basihyal, and between the anterior end of the basihyal and the mouth epithelium. It is interesting to note that this is the site of the future dermentoglossum bone and tooth buds. Another intensely active zone of mesenchyme is that above and below the trabecula communis. In the latter zone the parasphenoid has its origin.



TEXT-FIG. 1. From the lower jaw of a 12-mm. trout. Incubation time 6 hours. The highest concentration of phosphatase in the mesenchymal nuclei is in the vicinity of the dentary. No tooth papillae are formed yet. The epithelium is negative. The dentary is not calcified. Cf. Text-fig. 2.

All text-figs. except diagrams are camera-lucida drawings of undecalcified sections treated by the Gomori (1939) method, unless otherwise stated. Areas of phosphatase activity are shown black.



TEXT-FIG. 2. From the lower jaw of a 16-mm. trout. Incubation time 15 hours. The pair of Meckel's cartilages are just separated. The dermodentary is very close to the cartilage but not in apposition. Note the positive reaction of the mesenchymal nuclei and of the epithelium in the region of the tooth papilla. The cartilage matrix is slightly positive. The dentary is calcified.

B. Skeletal Tissues

In embryos of about 17 mm. the chondrocranium has almost reached its full development. The trabeculae, and the anterior part of the parachordals, display little or no phosphatase activity except in the dorsal perichondrium, and this applies to all subsequent stages. These parts do not ossify.

There is a strongly positive reaction of the cartilage matrix in apposition to the notochord, in the region of the future basioccipital. The auditory capsules display little or no phosphatase apart from the perichondrium.

Splanchnocranium. The extreme tip of Meckel's cartilage is still not calcified. It shows phosphatase in the nuclei and also some in the matrix, especially at the periphery, where a thin layer of osteoid, the first sign of the autodontary, can be seen. The dermodontary is first visible at a level where the two cartilages just separate (Text-fig. 2). This stage shows the earliest calcification in the dentary. It is interesting to observe that the ground substance of the bone here contains phosphatase (visualized in decalcified sections), whereas in the older specimens this is rarely the case. Only the central portion of the bone is calcified as shown by treatment with silver nitrate. A rim of osteoid remains at the periphery of all growing bones. In Text-fig. 4 Meckel's cartilage and the dentary are cut longitudinally. Calcification decreases in intensity towards the distal end of the bone, which consists of a shread of uncalcified osteoid. The mesenchymal cells and fibres surrounding the dentary are strongly positive in this region. Meckel's cartilage has positive nuclei throughout but extracellular phosphatase only at the anterior end, i.e. where the dentary is in apposition to it. There are no new developments in the other branchial cartilages.

Membrane Bone. The dermodontary has already been described and its appearance is typical for all membrane bones at an early stage in their formation. The appearance of the maxilla is as at Stage 1. The parasphenoid is now seen ventral to the trabecula communis. With regard to the presence of calcium salts and phosphatase it resembles the early dermodontary.

Summary of Stage 2

The general distribution of phosphatase does not differ greatly from that at Stage 1. The perichondrium of some cartilages is strongly positive. Extracellular phosphatase occurs in the cartilage matrix where perichondral osteoid is present or about to be formed, e.g. at the anterior end of Meckel's cartilage. The first stages of calcification are observed in membrane bones.

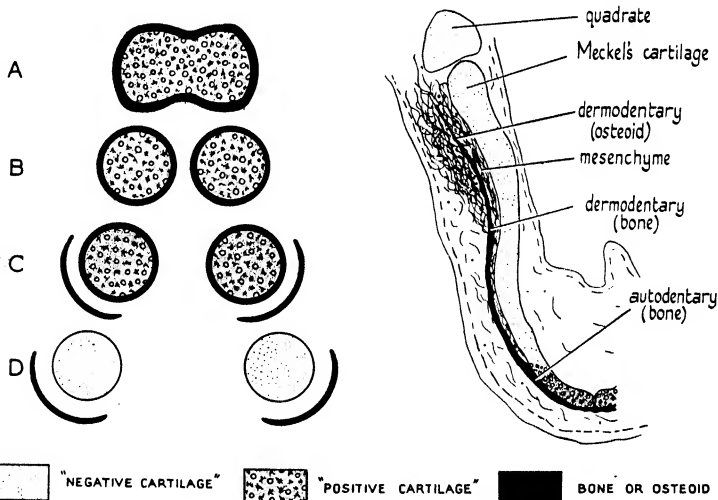
Stage 3 (20 mm.)

A. General Distribution of Phosphatase

The appearance of the central nervous system is unchanged. The retina displays a well-localized zone of high phosphatase activity in the nuclei of the light-receptive cells. The reaction is somewhat weaker at the periphery of the retina than in the central region. The lens epithelium is positive. The

sensory epithelium of the nasal grooves has positive nuclei, and the fine cilia protruding from the olfactory cells stain heavily. The epithelium of the mouth and skin is negative.

Phosphatase in the mesenchyme is localized in the zones described for the previous stage. Tooth buds have now appeared at the angles of the mouth and in the strongly positive zone above the basihyal. The epithelium is



TEXT-FIG. 3

TEXT-FIG. 4

TEXT-FIG. 3. Diagrammatic representation of transverse sections through Meckel's cartilage and dentary in different regions. A. Proximal tip. Cartilages fused and surrounded by the autodontary cartilages of 'positive type'. B. Cartilages just separated. 'Positive type'. C. Slightly distal to B. Proximal part of dermodentary overlapping with posterior part of autodontary. Cartilage still 'positive'. D. Distal to C only the dermodentary is seen. Cartilage of 'negative type'.

TEXT-FIG. 4. Diagrammatic representation of a longitudinal section through the lower jaw of a 16-mm. trout showing the distribution of phosphatase in Meckel's cartilage and the dentary. The latter is calcified only in its anterior portion. The posterior portion consists of osteoid and is partly obscured by strongly positive mesenchyme.

The key to the shading applies to all subsequent figures in which a diagrammatic style is used.

thrown into folds and papillae of the strongly staining mesenchyme project into it.

As in previous stages the mesenchyme surrounding the growing membrane bones is strongly positive.

B. Skeletal Tissues

The general distribution of phosphatase within the chondrocranium tends towards more localization of the enzyme in certain areas and reduction in others. Whereas in Stage 1 all cartilages have positive nuclei this is no longer the case; some are completely negative, others have very high concentrations

of phosphatase in both nuclei and matrix. As will be seen below this accumulation of enzyme can always be correlated with the development of a bony shell round the cartilage. Pl. 1, fig. 3, shows that the hyomandibula is positive in patches and the ceratohyal stains intensely. Portions of the chondrocranium which serve as attachment for muscles tend to give a positive reaction. There is a strongly staining region in the walls of the foramen of the facial nerve where the pro-otic bone can be seen in slightly older specimens (Stage 4).

Splanchnocranium. The extreme anterior end of Meckel's cartilage is now completely surrounded by a shell of bone, the mentomeckelian ossification, which merges into the autodontary posteriorly. The chondrocytes in the anterior portion show strongly positive nuclei and there is some phosphatase in the matrix, especially at the point where the pair of cartilages is just separating. The intensity of the phosphatase reaction decreases from the strong nuclear and extracellular reaction in the anterior tip to a weak nuclear reaction at a level where the cartilages are widely separated and the dentary is merging from its cartilage bone portion into the membranous portion. Distal to this the cartilage is completely negative apart from a few isolated nuclei chiefly at the periphery.

The appearance of the cartilage varies in the regions of different phosphatase activity: in the anterior portion the chondrocytes are large and spherical, their nuclei appear either completely black or granular, the cytoplasm gives a positive reaction, and the cells are surrounded by a rim of strongly staining ground substance, the rest of the matrix being moderately positive. In the eye region where the cartilage is negative the chondrocytes are arranged in longitudinal rows and are bilaterally compressed, in contrast to the spherical cells of the anterior zone. In the region of the optic chiasma the thick outer lamella of the dermodontary is fully calcified, while the inner thin lamella consists of uncalcified or feebly calcified osteoid. Both parts are embedded in strongly positive mesenchyme.

The Pterygoquadrate. The chondrocyte nuclei of the pterygoid process are moderately positive on the dorsal and external surface, i.e. where the metapterygoid bone is due to develop. In some of the specimens the endopterygoid (membrane bone) is seen as a thin layer of osteoid central to the inner perichondrium of the pterygoid process. It is not yet calcified and gives a positive phosphatase reaction.

In the region of its articulation with Meckel's cartilage and with the hyosymplectic cartilage the appearance of the quadrate process changes to that described for the anterior portion of Meckel's cartilage, and this is typical for all ossifying zones. The extracellular phosphatase activity is most marked near the lateral edge of the cartilage where a broad zone of feebly calcified osteoid, the first sign of the quadrate bone, can now be seen (Pl. 1, fig. 4). Phosphatase is never found in the matrix of cartilages which have no perichondral layer of osteoid or bone. Thus the characteristic features of ossifying cartilage in the trout are: (a) Greatly enlarged spherical cells with round nuclei, which later appear to degenerate and stain only faintly with haematoxylin.

These cells have very marked phosphatase activity. (b) The presence of phosphatase in the cartilage matrix, especially in the newly secreted ground substance round the chondrocytes which therefore appear to have black 'haloes'. The matrix is strongly basiphilic and in staining reaction resembles mammalian hypertrophic cartilage. However, no calcification of the matrix itself has been observed.

Since this type of cartilage is constantly met with, and always in conjunction with perichondral bone formation, it will be briefly referred to as 'positive cartilage', the above characteristics (including bone or osteoid) being implied in that expression. The term will not be used to describe cartilage containing phosphatase in the cells and perichondrium only. In diagrams of sections 'positive cartilage' is represented by areas with black rings, whereas 'negative cartilage' is stippled. Bone or osteoid is shaded. Thus the appearance of Meckel's cartilage described on p. 191 may be represented by a series of diagrams shown in Text-fig. 3.

In specimens of 15 mm. or over all cartilages fall into one of the following categories: (a) cartilages displaying no phosphatase activity whatever, (b) cartilages displaying some phosphatase in nuclei and perichondrium, and (c) cartilages with considerable phosphatase activity in cells, perichondrium, and matrix ('positive type'). Only the latter are ossifying.

The Hyoid Arch. The anterior zone of the symplectic process has the same appearance as the quadrate in this region, i.e. the perichondrium and chondrocyte nuclei are positive. Slightly distal to this there is an abrupt transition to the 'positive type' of cartilage. This is the first sign of the symplectic bone which ossifies in the distal portion of the hyosymplectic cartilage. Pl. 1, fig. 5, shows the posterior end of the quadrate and immediately below it the hyosymplectic cartilage with its layer of bone. The contrast between the adjacent cartilages is very marked.

The hyomandibular portion of the cartilage is not yet calcified but shows a thin perichondral lamella of pre-osseous tissue. The perichondrium is strongly positive especially where the cartilage is pierced by the hyomandibular branch of the facial nerve. The chondrocyte nuclei are positive throughout. A few enlarged cells of the type found in ossifying cartilages are seen and are surrounded by positively staining 'haloes' of ground substance. The mesenchyme enclosing the pre-opercular bone is intensely positive.

Other Branchial Cartilages. The ceratohyal is partly surrounded by a thin shell of bone (the epihyal) and presents the usual picture of an ossifying cartilage. No other cartilages have extracellular phosphatase, but some display positive nuclei and perichondrium.

Membrane Bone. The dermodontary has been described in conjunction with Meckel's cartilage. The maxilla is now heavily calcified especially in its anterior portion and is best studied in decalcified sections where its relationship to the surrounding mesenchyme is more clearly seen. It may be described in some detail as typical of membrane bone at this stage of development. In sections incubated for 14-16 hours the very heavy deposit of calcium salts

obscures the individual cells since the mesenchyme at the angles of the mouth is strongly positive. But in sections incubated for 4-6 hours only, the cells close to the maxilla (or other membrane bone studied) are positive, hence these cells have the highest concentration of phosphatase. The bone itself is negative after this incubation time but may appear grey after 18 hours. Three types of cells can be distinguished in the vicinity of the bone: long fibroblasts with narrow nuclei, large oval cells with prominent nuclei, and polymorphous cells of an intermediate size. The latter make up the bulk of the mesenchymal cells. The large cells are found close to the bone. They may be osteoblasts. The phosphatase content of these cell types is very variable. The cytoplasm only displays phosphatase after very long incubation times. The fibroblast nuclei stain deep black, most other nuclei appear granular. Nucleoli and nuclear membranes are prominent in all cell types. Text-fig. 5 shows a typical field of membrane bone and surrounding cells. In the bone itself two regions of different refractive index can sometimes be distinguished: a central and peripheral zone. In stained preparations, too, the heterogeneous nature of the ground substance becomes evident, the older ground substance having a different appearance from that newly secreted.

Summary of Stage 3

Ossifying cartilages can be distinguished from non-ossifying ones by the presence of high concentrations of phosphatase in the matrix as well as in the enlarged chondrocytes. In non-ossifying cartilages phosphatase, if present, is confined to the nuclei. Membrane bones display phosphatase only in the newly formed portions.

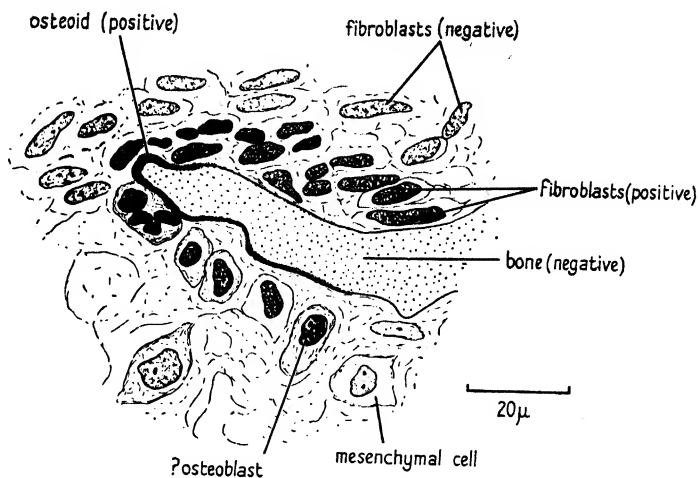
Stage 4 (21-3 mm.)

A. General Distribution of Phosphatase

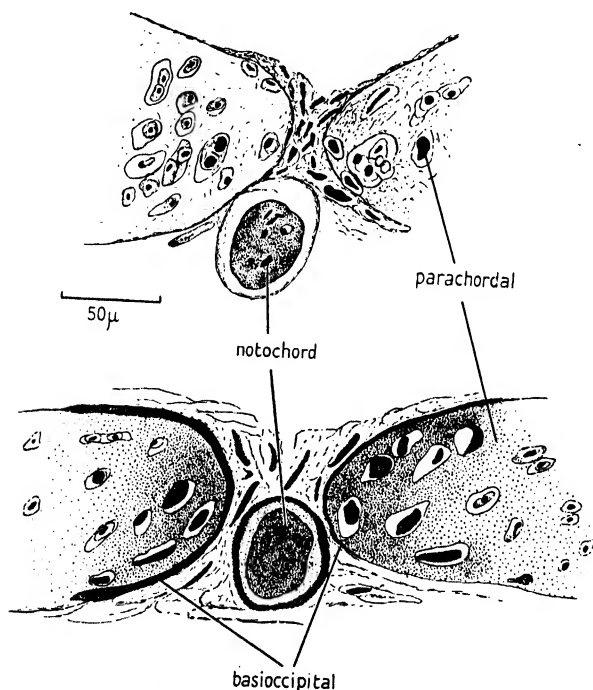
The decline of nuclear phosphatase in tissues such as muscle, peripheral nerves, mesenchyme, and non-calcifying cartilage, already noted at the previous stage, continues. The skin and mouth epithelium, except at the tip of the lower jaw, remain negative. The anterior region of the brain, the retina, lens epithelium, and the nasal mucosae are strongly positive. The reaction becomes weaker in the mid-brain, the rest of the central nervous system being only feeble positive.

B. Skeletal Tissues

At the exit of the facial nerve, where the pro-otic is now seen in the form of two perichondral lamellae, the anterior wall of the auditory capsule is positive in cells and matrix. The bony lamellae, like all perichondral ossifications, are homogeneous and sharply separated from the cartilage as well as from the connective tissue. (Appearance similar to parachordals in Text-fig. 6.) A few small spindly shaped cells form the periosteum. Ossification of the lateral commissure and the walls of the foramen for the trigeminal nerve has also begun.

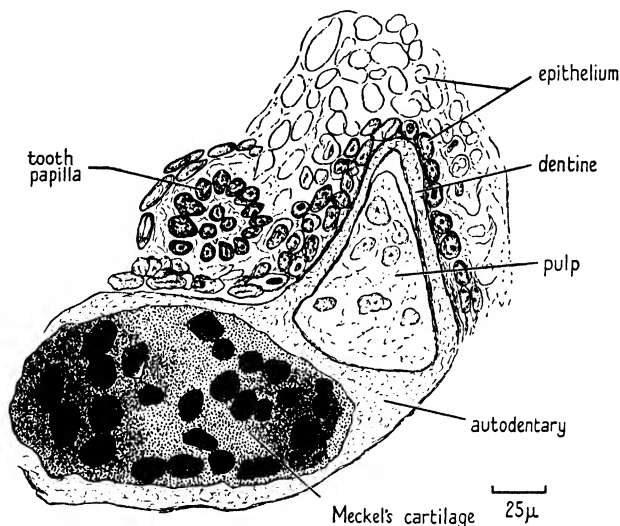


TEXT-FIG. 5. Membrane bone and associated cell types of a 20-mm. trout. Decalcified section. Incubation time 15 hours. Note that only the growing-tip contains phosphatase.



TEXT-FIG. 6. Transverse sections through the notochord, parachordals, and basi-occipital of a 23-mm. trout. Incubation time 15 hours. Section (b) is slightly distal to (a). The perichondral lamellae of the basi-occipital are seen in (b) and the cartilage matrix displays phosphatase activity in that region.

The parachordals in the region of the myodome between the pro-otic and the basi-occipital are negative. The anterior tip of the notochord is strongly positive and a little farther back the central part of the parachordals also displays phosphatase activity, first in the nuclei only, then throughout the matrix. The space between the parachordals contains strongly positive connective tissue (Text-fig. 6). This, according to Schleip, ossifies later. The basi-occipital is now seen in the form of two perichondral lamellae round the



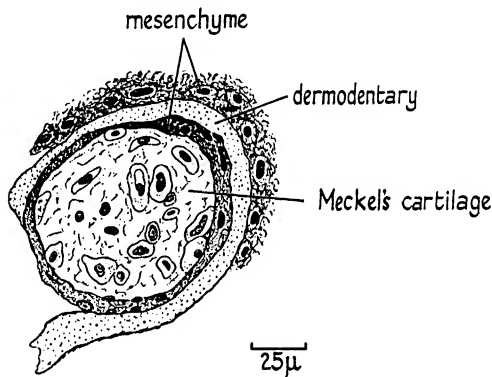
TEXT-FIG. 7. From the lower jaw of a 22-mm. trout. Decalcified section. Incubation time 15 hours. Anterior tip of Meckel's cartilage and the autodontary. The cartilage is strongly positive in nuclei and matrix. The bone is negative.

central ends of the parachordals (Text-fig. 6). Still farther back the parachordals are adjacent to the notochord and the bony lamellae continuous with the notochord sheath.

The *splanchnocranium* of this stage shows very clearly the correlation between perichondral bone and *extracellular* phosphatase and the absence of any correlation between (a) perichondral bone and *nuclear* phosphatase, and (b) membrane bone and phosphatase within adjacent cartilages. The reaction in the matrix is never as intense as the reaction given by the enlarged chondrocytes in the ossifying zone.

The appearance of Meckel's cartilage is as described for the previous stage. Teeth at various degrees of development are present. The mesenchyme round the tooth buds and beneath the mouth epithelium is strongly positive (Text-fig. 7). As the autodontary decreases in thickness posteriorly, the concentration of phosphatase in Meckel's cartilage gets less as already

described for the younger specimens. In Text-fig. 8 there is a thin layer of mesenchyme between cartilage and bone, and the extracellular phosphatase in the former has completely disappeared. The nuclei are still positive but the intensity of this reaction diminishes and finally the cartilage is completely negative in its distal part, although the dentary approaches it again. But there is always some mesenchyme between the two structures. Thus it is seen again that mere proximity of bone is not correlated with a positive phosphatase reaction in the cartilage, whereas actual apposition of bone is associated with a marked staining of chondrocytes as well as matrix.



TEXT-FIG. 8. From the lower jaw of a 22-mm. trout. Decalcified section. Incubation time 15 hours. Shows a more distal region of Meckel's cartilage than Text-fig. 7. The dentary is here separated from the cartilage by connective tissue. The cartilage matrix is negative. In Text-figs. 7 and 8 the bone itself shows no phosphatase activity.

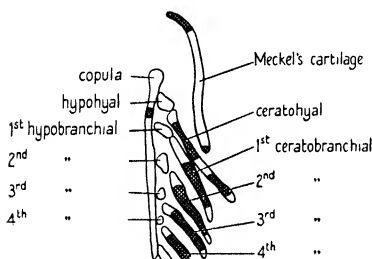
This is also illustrated by the pterygoquadrate and the hyomandibula. Ossification in the other branchial cartilages has made marked progress since the previous stage examined. The distribution of extracellular phosphatase and perichondral bone is shown in Text-fig. 9. The two perichondral ossifications of the ceratohyal cartilage have increased in thickness and are heavily calcified. The ceratobranchials display phosphatase activity and perichondral bone in their central portions only. Their anterior and posterior tips remain negative. Hence the appearance in transverse sections varies according to the level. The hypobranchials are negative throughout and have no osteoid or bone. Nuclear phosphatase is present in most of the visceral cartilages and the perichondrium is positive in some regions especially on the dorsal surface of the copula where the dermentoglossum (membrane bone) is now developing ventrally to a set of teeth to which it becomes attached. The mesenchyme in this zone is strongly positive as has already been pointed out at earlier stages. A detailed description of the chondrocranium is given in my thesis (Lorch, 1948).

Membrane Bone. A number of new membrane bones have now taken shape and some of the bones previously described are beginning to assume the

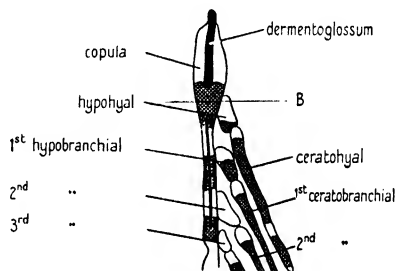
appearance of a meshwork of ground substance interspersed with cells which are penetrating from the surrounding mesenchyme. The newly formed bones, whether they arise in connexion with the lateral line canals such as the nasals and frontals, or as flat plates to be fused later with teeth such as the vomer and pre-maxilla, do not differ from the description given for developing membrane bones of earlier stages.

Summary of Stage 4

There is little or no phosphatase in non-skeletal tissues with the exception of the central nervous system and some sensory organs which remain strongly positive. All ossifying cartilages display nuclear as well as extracellular phosphatase, while the matrix of non-ossifying cartilages is always negative. The perichondrium of most cartilages displays enzyme activity. The phosphatase content of a cartilage is in no way correlated with the proximity of a membrane bone. The latter are positive at the growing-points.



TEXT-FIG. 9



TEXT-FIG. 10

TEXT-FIG. 9. Diagram of the principal visceral cartilages at the 23-mm. stage based on serial sections showing phosphatase distribution. (Modified after de Beer's reconstruction of a 16-mm. embryo.) Dorsal view of right side. Areas of cartilage bone formation and extracellular phosphatase are shaded.

TEXT-FIG. 10. Diagram of the principal visceral cartilages of a 38-mm. trout. Shading indicates extracellular phosphatase. Membrane bone is shown black. The level of the section shown in Pl. I, fig. 6, is indicated by the line B.

Stage 5 (29-38 mm.)

From the point of view of phosphatase distribution the change from the 23-mm. embryo to the relatively mature state reached at 38 mm. is so gradual that it is best to describe the appearance of the oldest specimens so as to bring out major developments.

The following account is based on serial sections through a 38-mm. trout (about 3 months after hatching), which was decalcified for 5 hours. Sections were incubated for 6 or 16 hours, and counterstained with eosin. A 36-mm. specimen, cut undecalcified, served for comparison, especially of the calcified structures, but was found histologically inferior owing to the difficulty of cutting thin serial sections through such brittle material. With regard to the somewhat smaller specimens, fairly good sections were obtained and the

two-colour technique, using gallamine blue for phosphatase, was found most useful, in that the sites both of calcification and of phosphatase activity could be visualized in the same section.

A. General Distribution of Phosphatase

The general distribution of phosphatase in non-skeletal tissues is mainly as described for Stage 4. The mesenchyme has changed somewhat in appearance in the 38-mm. specimens: fibres have developed between the cells and the latter may now be called connective tissue cells rather than undifferentiated mesenchyme. It has previously been noted that phosphatase in the mesenchyme was at first widespread but then tended to become localized in the regions of membrane bone or tooth development. There seems to be a slight reversal of this tendency: areas of connective tissue not in immediate contact with calcifying structures are found to be strongly positive in the cells—many of which have processes—and in the fibres. Since there is an extensive formation of fibres at this stage the reappearance of phosphatase in the connective tissue may be related to collagen formation.

B. Skeletal Tissues

The classification of cartilage described for Stage 4 applies here equally.

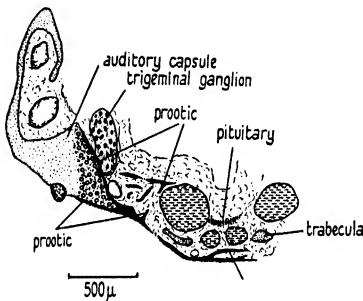
The trabecula communis and the paired trabeculae are negative. The anterior parts of the parachordals remain strongly positive in the region of the pro-otic. This bone has developed extensively and comprises perichondral lamellae of the anterior end of the parachordals, the anterior wall of the auditory capsules, the roof of the myodome, and the lateral commissure. The inner lamella of the pro-otic lies below the cerebrum and the outer represents ossification of the major part of the base of the skull.

The development of the pro-otic is a typical example of perichondral and endochondral ossification with participation of ossifying connective tissue and will therefore be described in some detail.

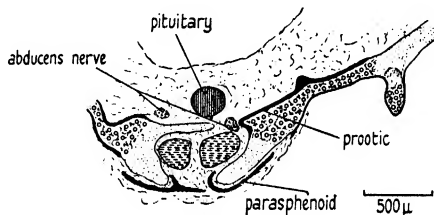
With regard to the distribution of phosphatase in the region of the pro-otic, the same principles hold as for cartilage bone formation elsewhere in so far as the pro-otic is represented by perichondral lamellae. Where it is formed by ossification of the membranes of the brain, the latter display strong phosphatase activity. The bone itself—whether perichondral or membranous—only displays very slight phosphatase activity at the edges. Examples of transverse sections through the region of the pro-otic will show its complicated structure and its relationship to cartilage, membranes, and to the parasphenoid bone: Text-fig. 11 shows the left half of the anterior region of the myodome and the exit of the trigeminal nerve. The pro-otic is seen as a perichondral lamella of the anterior auditory capsule and extending inwards towards the parasphenoid. Above this extension, the pro-otic has formed a network of interlacing trabeculae connecting dorsally with the ossified membranous base of the brain and forming the lateral wall of the myodome. The nuclei of the cells filling the spaces between the bony

trabeculae are positive, as are also the nuclei of all other tissues in the region shown: ganglion cells of the trigeminal nerve nucleus, Schwann cells, nuclei of striated muscle, connective tissue cells, and erythrocytes. The only marked concentration of extracellular phosphatase is seen in the ossifying cartilage.

Slightly distal to the level shown in Text-fig. 11, the lateral commissure becomes visible. It is strongly positive in its middle portion where it is covered dorsally and ventrally by perichondral lamellae of the pro-otic. But its central tip is negative. The parasphenoid extends below this part of the lateral commissure but is separated from it by the perichondrium.



TEXT-FIG. 11



TEXT-FIG. 12

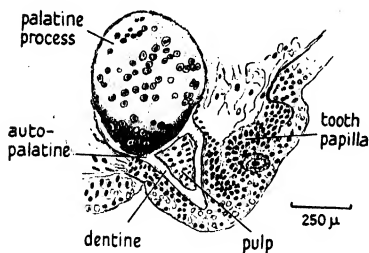
TEXT-FIGS. 11 and 12. Diagrammatic cross-sections through the posterior myodome region of a 38-mm. trout to show the distribution of phosphatase in the cartilage and its relation to associated bones. Key to shading as in Text-fig. 3. Description in text.

In Text-fig. 12 the cartilaginous roof of the myodome is seen. Its cells are arranged in transverse rows. At the point shown only the perichondrium and the chondrocyte nuclei have phosphatase activity. But distal to this region, where the pro-otic grows over the cartilage, the latter is strongly positive. The entry of the abducens nerve into the myodome is seen. The lateral walls of the myodome are formed by the anterior ends of the parachordals. Ventrally the parasphenoid is seen to curve inwards and partially surrounds the parachordals. The latter are positive only where the pro-otic has formed a perichondral lamella.

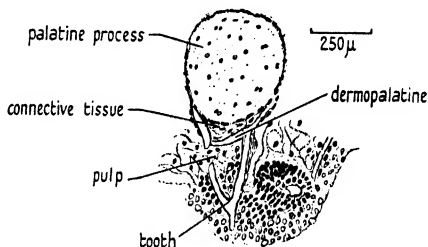
The extreme tip of the notochord is strongly positive and surrounded by an ossified sheath which is continuous with the basi-occipital. More distally only the notochord sheath contains phosphatase, the central tissue being negative. The basi-occipital forms perichondral lamellae dorsally and ventrally to the parachordals which are now positive. The ventral wall of the posterior semicircular canal is negative but becomes positive laterally where the exoccipital bone is developing. A bony lamella lying in the strongly positive membranes covering the ventrolateral aspect of the brain connects the basi-occipital with the roof of the brain. The exoccipital is well developed round the jugular foramen and the cartilage in that region is positive.

Splanchnocranium. Calcification of the autodontary although intense in places is not complete. The growing-surfaces are free from calcium salts. The bone and osteoid are relatively poor in phosphatase, but the connective tissue surrounding the dentary is strongly positive especially at the growing-tips. The phosphatase seems to be mainly nuclear, the cytoplasm of the osteoblasts and connective tissue cells is negative. But in areas of very great phosphatase activity some positive fibrils are seen.

The middle portion of Meckel's cartilage contains no extracellular phosphatase in these older specimens but the chondrocyte nuclei and the perichondral fibroblasts are positive in some areas. In the oldest fish examined



TEXT-FIG. 13



TEXT-FIG. 14

TEXT-FIGS. 13 and 14. From decalcified sections through a 38-mm. trout. Incubation time 6 hours. The palatine process of the pterygoquadrate is shown at two levels. In Fig. 13 the palatine bone forms a perichondral lamella and the ventral region of the cartilage is positive. In Fig. 14 the palatine bone is separated from the cartilage which has only nuclear phosphatase.

(36–8 mm.) Meckel's cartilage shows two regions of phosphatase activity in its posterior portion: just before articulation with the quadrate there is a patch of positive cartilage which at first sight seems to have no relation to any perichondral bone since it is mainly in the centre of the cartilage. But closer inspection of consecutive sections shows that in some places the angular does come into direct contact with the cartilage laterally. The angular, according to Haines (1937), has taken the place of the articular in most teleosts. Like the dentary it has a cartilage and a membrane bone portion, the former being regarded by previous authors as the articular (autoarticular of Böker).

The Pterygoquadrate. At all previous stages the palatine process of the pterygoquadrate was completely negative or only showed slight phosphatase activity in nuclei and perichondrium. The palatine bone was then separated from the cartilage by a thin layer of connective tissue. In the 38-mm. specimen a different picture is obtained: anteriorly the palatine is composed of a very thin perichondral lamella and a membrane bone portion to which the teeth are fused. The two parts of the bone are partially separated by a very thin layer of connective tissue but are fused laterally. The cartilage is positive at its ventral edge, i.e. where the autopalatine is in apposition (Text-fig. 13). The dermopalatine extends farther backwards than the autopalatine. Hence

more distal sections show no perichondral lamella and also no phosphatase in the cartilage (Text-fig. 14). The dermopalatine is separated from the palatine process by a thin layer of connective tissue, the nuclei of which are positive. In the pterygoid region the cartilage is negative and the perichondrium positive, being in close relation to the two membrane bones (ecto- and endopterygoid) of that zone.

The distribution of phosphatase in the quadrate is similar to that at the previous stage. The tendinous tissue connecting the quadrate with the symplectic is strongly positive. It is said to ossify.

The symplectic bone is becoming thicker at the expense of the central cartilage which is very strongly positive and shows much-enlarged cells with degenerating nuclei. There is resorption of cartilage and formation of marrow spaces near the foramen for the hyomandibular branch of the facial nerve. The cartilage in that region is strongly positive. The nuclei of the perichondral and periosteal cells show phosphatase activity; Schwann cell nuclei as well as those of bone-marrow cells are also positive. In all the branchial cartilages where perichondral bone is formed there are well-defined zones of extracellular phosphatase. Areas free from bone have no phosphatase. The degree of phosphatase activity does not seem to depend on the thickness of the perichondral bone. Text-fig. 10 is a diagram of part of the branchial skeleton showing the position of 'positive cartilage' in a 38-mm. trout. This is based on serial sections, an example of which is given in Pl. 1, fig. 6. With regard to the two membrane bones dorsal to the copula the following point is again illustrated: there is no correlation between a positive reaction of the cartilage and the proximity of a membrane bone.

Membrane Bone. In the oldest specimens examined, the histological structure of the membrane bones is now clearly that of an 'adult' trout. There is no sharp transition to adult condition such as is found, for instance, in mammalian long bones where the epiphyseal cartilage is replaced by bone. Since fish continue growing throughout their life if conditions are favourable, the term 'adult' must be applied with reservations. The chief difference between teleost bone and mammalian bone lies in the relative scarcity of osteocytes. According to Kölliker (1859) these are entirely missing in some teleosts. He called the acellular tissue 'osteoid', a term which I have used here to describe *uncalcified* bone irrespective of the presence of osteocytes. Schmid-Monnard (1883) admits that the primary bony lamella is a structureless acellular mass, but in adult bones osteocytes are occasionally seen. Stéphan (1900) points out that both acellular and cellular bone is found in teleosts and that the former invariably consists of thin lamellae through which nutrients could diffuse, thus eliminating the necessity for a vascular system such as the Haversian systems of mammalian compact bone. The last observation is confirmed by the present series: thin, bony lamellae—whether perichondral or membrane bone—are devoid of cells. Many membrane bones, e.g. the nasals and frontals, remain thin plates and only very few osteocytes could be found in such bones. But bones which rapidly increase in bulk such

as the pre-maxillae and maxillae and the perichondral ossification of the symplectic show a fair number of cells within the ground substance (Pl. 2, fig. 1). Marrow spaces are seen in some bones. The ground substance shows lines separating the older from the more recently secreted matrix. Near the outer limit of the bone there is often a black line indicating phosphatase activity. Osteoblasts, if present, are ranged outside the osteoid layer and are usually strongly positive, as for instance at the distal end of the maxilla (Pl. 2, fig. 4). The same relationship as has just been described for bone, osteoid, and osteoblasts exists between calcified dentine, uncalcified dentine, and odontoblasts.

The relationship between membrane bone and cartilage at this stage is illustrated by the vomer in the region where it forms a continuous lamella roughly following the shape of the cartilage from which it is separated by a layer of very cellular, intensely positive connective tissue (Pl. 2, figs. 2 and 3). Below this bony lamella the connective tissue is less cellular, but the nuclei also display strong phosphatase activity.

The frontal may be mentioned here as a typical 'canal bone'. It consists of a bony tube surrounding a lateral line canal and a flat lamella extending inwards towards the mid-dorsal line. Another shorter process extends outwards from the canal. The bony plates are separated from the cartilage of the tectum cranii by a very thin layer of tissue which is both perichondrium and periosteum. It is never thicker than three layers of fibroblasts. As has constantly been noted for connective tissue between cartilage and bone, it has marked phosphatase activity. The underlying cartilage is negative. The frontal bone itself is negative in its thicker (peripheral) portions. It tapers to a thin end centrally and here, i.e. at the growing-point, it displays the 'positive lines'. The walls of the tube surrounding the lateral line canal are somewhat thicker than the flat part of the frontal and occasional osteocytes are seen. Again the growing (dorsal) tips of the bone have some phosphatase peripherally and accumulations of osteoblasts are seen. These are strongly positive, but so are most other connective tissue cells and fibres. Groups of the large round cells, noted also in connexion with the pre-opercular, are seen at the junction of the canal bone portions and the flat part of the frontal. These cells are devoid of phosphatase except for the nucleoli which stain faintly. Minute fragments of bone are sometimes found between them, and these fragments, unlike the newly formed osteoid, are free from phosphatase. The large cells suggest a possible osteolytic function. Although the connective tissue between bone and skin epithelium is strongly positive, the epithelial cells themselves display on the whole no phosphatase activity.

Summary of Stage 5

Further examples of the correlation between perichondral ossification and extracellular phosphatase are given. The structure of some membrane bones is described.

DISCUSSION

The following points emerge from the study of the distribution of phosphatase and 'bone salts' in growing trout.

In the early stages of development the enzyme is widely distributed and is on the whole confined to the nuclei. As differentiation proceeds phosphatase becomes more concentrated at sites of bone or fibre formation, while the nuclei of the undifferentiated mesenchyme and non-calcifying cartilage display less phosphatase activity. With the appearance of perichondral osteoid the chondrocytes undergo a marked change in appearance and, simultaneously, phosphatase activity spreads from the cells to the matrix. The change is reminiscent of that observed in mammalian hypertrophic cartilage. It is significant that bone is never formed in the absence of extracellular phosphatase. This observation is in agreement with that previously made on mammalian bones (Lorch, 1947). It must be noted that the maximum concentration of phosphatase in the cartilage occurs before there is any sign of calcification and at sites which do not themselves calcify. The pre-osseous substance seems to contain very little phosphatase. However, the perichondrium (which becomes the periosteum) is always strongly positive. A speculation regarding the source of phosphatase in cartilage bone formation is of interest. In mammalian endochondral ossification the enzyme is said to be derived from osteoblasts as well as hypertrophic cartilage cells. In the trout, osteoblasts are not prominent and are especially rare in connexion with perichondral bone formation. Therefore the most likely sources of phosphatase are the enlarged cartilage cells which may secrete the enzyme into the matrix with which the bone is in contact. The strongly positive reaction of these cells and the adjacent matrix favours this view. The fact that the cartilage itself does not calcify at the stages examined is surprising, but may be a necessary condition for the diffusion of the enzyme from the cells to the periphery.

In membrane bone formation increased phosphatase activity was also noted in the mesenchyme well before the onset of calcification. The enzyme occurred in fibres as well as cells, but the change from purely intracellular to extracellular phosphatase was not as marked here as in cartilage bone formation.

However close a membrane bone is to a cartilage, the latter never displays phosphatase in the matrix, unless it is itself ossifying. Hence the two types of bone can easily be distinguished by means of the Gomori method.

The absence of phosphatase from the calcifying osteoid and the fact that calcification starts at the centre, whereas the highest concentration of enzyme is found at the periphery, may seem surprising. However, if it be considered that osteoid is a dense avascular tissue surrounded on all sides by a zone of high phosphatase activity, it seems likely that the inorganic phosphate liberated at the periphery tends to accumulate within the osteoid and so a high level of phosphate ions may be reached without the presence of phosphatase at the actual site of calcification.

As calcification proceeds, new layers of osteoid are formed at the periphery. Once the bone is well defined, its phosphatase content is usually very low, except at the growing-tips and edges. Very few cells are enclosed in the bony matrix and they contain no phosphatase. The phosphatase content of the mesenchymal cells and fibres stays high as ossification proceeds although the bone itself may be quite negative.

The biochemical studies of phosphatase in fish, mentioned in the introduction, must necessarily deal with organs or parts of organs and no information regarding the distribution of phosphatase within a tissue or its intracellular distribution can be gained from them. The facts previously established may be briefly reviewed in the light of the present work: Roche and Bullinger (1939) found that 'phosphatase was present in all teleost bones examined and high concentrations of enzyme were present in scales and teeth'.

Roche and Collet (1940), working on the sardine, found that there was a seasonal increase in phosphatase activity in the *whole* skeleton during spring and early summer, i.e. when optimum conditions for growth prevail. They emphasize that this constitutes evidence for the physiological regulation of phosphatase activity in the skeleton as a whole, possibly by an endocrine mechanism. Without wishing to contradict this hypothesis for which there is independent evidence (Roche and Filippi, 1938), I think it should be pointed out that the increase of phosphatase activity in different parts of the skeleton may be 'simultaneous' when reckoned in terms of months, but histochemical studies of the ossification of the chondrocranium of the trout show that the increase of phosphatase activity of each cartilage—or portion of cartilage—is exactly correlated with the appearance of perichondral osteoid round the particular cartilage and in no way influenced by ossification in an adjacent cartilage or within the mesenchyme.

With regard to the distribution of phosphatase in the embryo generally a comparison with results obtained by Moog (1944) on chick embryos and by Horowitz (1942) on the heads of foetal rats is interesting.

According to Moog 'phosphatase persists as long as a tissue remains undifferentiated. As differentiation proceeds, phosphatase in some cases disappears and in others accumulates in higher concentrations than in the primitive phase.' Although the trout embryos examined did not include the very early stages of development comparable to Moog's chick embryos, her statement is on the whole confirmed and similar observations were made on the marine teleost *Cottus bubalis*, early stages of which were examined as a preliminary study (unpublished) to the present work.

Horowitz (1942) commences his study of phosphatase and glycogen with rat foetuses at the gill arch stage (13 days) and finds them 'devoid of phosphatase'. However, incubation was only carried out for 2 hours.

In 15-day foetuses Horowitz notes that prospective regions of calcification show a marked phosphatase activity; i.e. they become chemically differentiated before the occurrence of any morphological differentiation. This is in accord with the present results. Also the irregular distribution of phosphatase in the

central nervous system, and its high concentration in the linings of blood-vessels, in taste-buds, and in the lens epithelium are paralleled in the trout embryos.

With regard to ossifying cartilage Horowitz's results are in agreement with previous descriptions for other species: hypertrophic cartilage containing high concentrations of phosphatase in cells, matrix, and perichondrium is invariably associated with ossification.

It is seen that apart from minor differences, there is a striking parallelism between phosphatase distribution in developing embryos belonging to species as widely different as chicks, rats, and trout. It is therefore indeed likely, as suggested by Moog, that phosphatase plays a fundamental role in histogenesis, apart from its function in the development of calcified structures.

I should like to thank all those who have helped me by their advice and criticism, particularly Dr. J. F. Danielli for his encouragement throughout this work.

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SUMMARY

1. The histological and cytological distribution of alkaline phosphatase in developing trout has been studied with special reference to membrane and cartilage bone formation in the skull.
2. Nuclear phosphatase is widely distributed in the youngest stages examined, but decreases as differentiation proceeds.
3. Extracellular phosphatase is always associated with ossification or fibre formation.
4. No deposition of calcium salts in the absence of phosphatase was observed.
5. Alkaline phosphatase is probably connected with histogenesis in general apart from its special function in calcification.

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DESCRIPTION OF PLATES 1 AND 2

All figures are unretouched photomicrographs of undecalcified sections treated by the Gomori (1939) method, unless otherwise stated. Areas of phosphatase activity are shown black. No counterstain was used.

PLATE I

Fig. 1. Trabecula communis of a 12-mm. trout. Incubation time 6 hours. The chondrocyte nuclei are positive. Note the strong reaction of the mesenchyme dorsal to the trabecula.

Fig. 2. Quadrate and hyosymplectic cartilage of a 12-mm. trout. Incubation time 6 hours. Note the strongly staining mesenchyme lateral to the cartilages. This is the site of the future pre-opercular bone. Chondrocyte nuclei and perichondrium are positive.

Fig. 3. Transverse section through the anterior auditory region of a 20-mm. trout. Incubation time 2 hours. Note the intense reaction of the ceratohyal and the absence of phosphatase from the parachordals and auditory capsule. The hyomandibula shows patches of phosphatase activity.

Fig. 4. Quadrate process of a 20-mm. trout showing perichondral bone. Incubation time 2 hours. Note the strongly positive reaction of the matrix near the zone of ossification.

Fig. 5. Distal end of quadrate cartilage at its articulation with the symplectic process. 20-mm. trout. Incubation time 6 hours. Note the difference in appearance and phosphatase content between non-ossifying and ossifying cartilage: only the symplectic is surrounded by a layer of osteoid.

Fig. 6. Copula and hypohyals of a 38-mm. trout. Level B in Text-fig. 10. Decalcified transverse section, incubation time 6 hours. Counterstained with eosin. Note the positive reaction of the copula which has a shell of perichondral bone. The hypohyals are only positive ventrally where ossification is beginning.

PLATE 2

Fig. 1. Undecalcified premaxilla of a 30-mm. trout. Bone salts visualized as cobalt sulphide. *Not* incubated for phosphatase visualization. Note cell spaces in the bone.

Fig. 2. Trabecula communis and vomer of a 32-mm. trout. Incubation time 15 hours. Note the strongly positive connective tissue in the area of tooth formation.

Fig. 3. Detail from centre of previous figure. Note the clear zone of osteoid (free from both calcium salts and phosphatase) on both sides of the vomer. The perichondrium on the ventral aspect of the trabecula is strongly positive.

Fig. 4. Distal end of maxilla of a 29-mm. trout. Incubation time 4 hours. The maxilla is not calcified in this zone. It displays faint phosphatase activity. Osteoblasts are seen on both sides of the osteoid. The surrounding connective tissue is strongly positive.

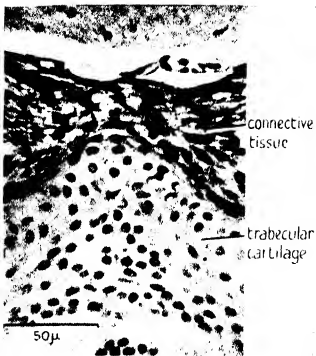


FIG. 1

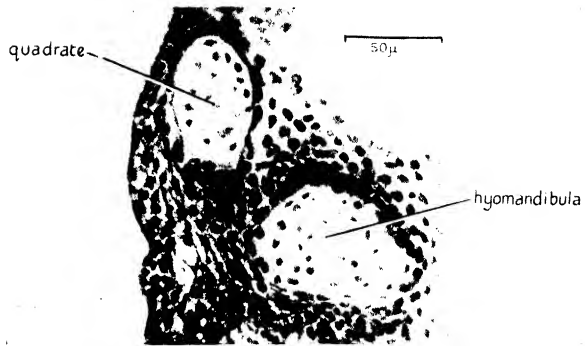


FIG. 2

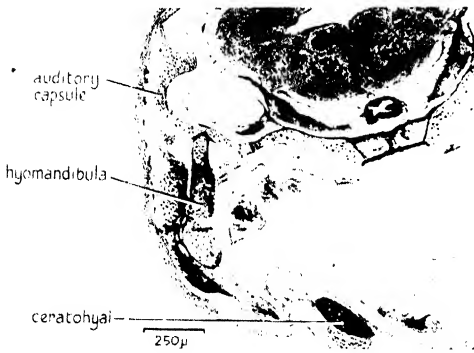


FIG. 3

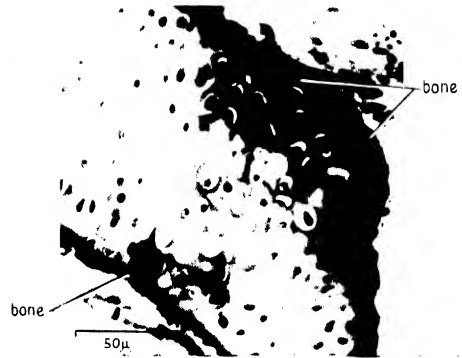


FIG. 4



FIG. 5

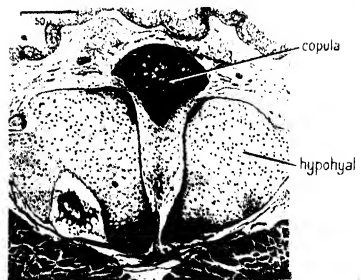


FIG. 6

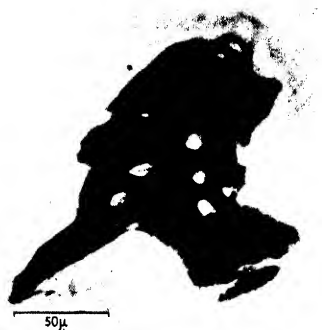


FIG. 1

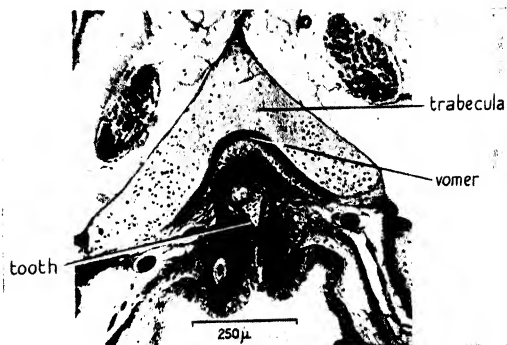


FIG. 2

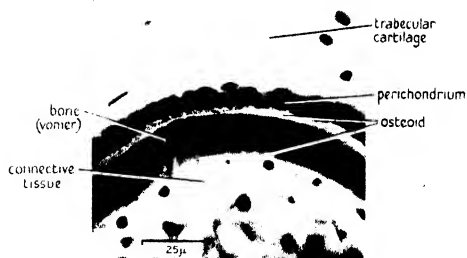


FIG. 3

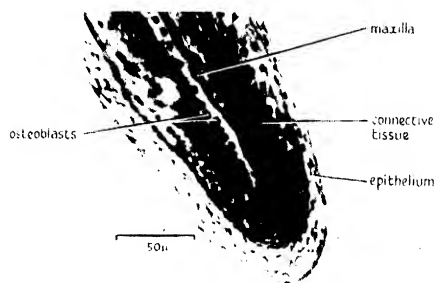


FIG. 4

Location of Absorbed Carcinogens within the Amphibian Cell

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INTRODUCTION

SOME of the polycyclic hydrocarbons derived from phenanthrene are biologically active in a number of different ways. Not only are they, as is well known, capable of causing the production of cancers and simulating the action of sex hormones, but they can also evoke neural tissue in the amphibian embryo (Waddington and D. M. Needham, 1935), and exert a rather feeble mutagenic activity (Demerec, 1947, 1948; Strong, 1947; Carr, 1947).

In none of these connexions have we a clear idea of the mechanism by which the effect is produced. This paper is, in the first instance, concerned to throw further light on the interaction between the carcinogenic substances and the embryonic amphibian cells on which they exhibit the activity of evocation. There are several questions which present themselves in this connexion. One is the relation between the carcinogen evocators and the substance, which may be called the 'natural evocator', which is presumably responsible for stimulating the ectoderm to differentiate into neural tissue during normal development. The demonstration of Waddington, Needham, and Brachet (1936) that evocation could be produced by treatment with methylene blue proved that evocation can be brought about by substances which are certainly not present in normal eggs. Waddington, Needham, and Brachet pointed out that such substances might act by killing a few of the neighbouring cells, thus releasing their stores of the natural evocator, which stimulates the surviving cells to neural differentiation. Holtfreter (1944*b*, 1945) and others following him at first suggested that all evocations by chemical implants were mediated by this mechanism. But recently Holtfreter (1948) appears to have convinced himself of the truth of the contention of Waddington, Needham, and Brachet that evocation is possible even when no signs of necrosis can be detected; and he seems ready to accept their suggestion that non-natural evocators may act, in some manner less drastic than killing, so as to release a store of natural evocator which is contained within

the ectoderm cells and which then brings about an induction. It is very possible that the carcinogenic hydrocarbons owe their evocating power to an action of this kind. Alternatively, they might be related chemically to the natural evocator, about whose properties we are still completely in the dark. Waddington (1940) and Needham (1942) have suggested that the low dosage in which the evocating activity is manifested offers some support for this suggestion; but they admit that too much weight cannot be placed on the argument.

It was not to be expected that, from observations of the kind to be recorded in this paper, any direct evidence would be forthcoming on the general question of whether the carcinogens acted as substitutes for the natural evocator or in a secondary way as releasers of it. But it was anticipated that some light might be thrown on their relation to one of the other substances which have been claimed to be the natural evocator. Fischer, Wehmeier, and Jühling (1933) first showed that preparations of ribonucleic acid can act as evocators, but they did not exclude the possibility that this was a result of producing localized necrosis. Brachet (references and discussion in 1947) has confirmed the result, using purified preparations; and has also presented evidence to show that when dead tissue is digested with ribonuclease, it loses its evocating power. He argues that the synthesis of cell-protein is carried out mainly at the ultra-centrifugeable ribonucleoprotein granules in the cytoplasm, to which Claude (1941) had first directed attention; and he suggests that evocation is primarily a reaction which takes place at these granules. He appears to believe that the natural evocator substance is actually ribonucleic acid, which diffuses from the roof of the primitive gut into the overlying ectoderm and leads to an increase in concentration there of these cytoplasmic granules. And he further suggests that the activity of the carcinogens is due to their property of becoming attached to the granules.

In making the last suggestion, Brachet bases himself on the observations of Graffi (1939, 1940). This author studied the accumulation of various polycyclic hydrocarbons within the cells of normal and malignant tissues of the mouse. The technique used was to render the hydrocarbons water soluble by treatment with glycerine and blood-serum, to expose the cells to aqueous solutions containing up to about one part of hydrocarbon in 40,000, and to study the location of the chemical within the cell by observing the fluorescence of the cell constituents when illuminated with ultraviolet light. Graffi found that the hydrocarbons become attached to cytoplasmic granules. He speaks of these as 'lipocondria' and 'mitochondria', but it is notoriously difficult to be certain exactly what various authors mean by these terms; and it was perhaps not out of the question that Brachet might have been correct in suggesting that some of the granules which accumulate the substances may be identical with the ribonucleoprotein granules (which may be called 'microsomes') to which he attributes synthetic activity.

Although Brachet's theory provides an attractive way of envisaging the mode of action of hydrocarbons in both carcinogenesis and evocation, it is

clear that its observational basis is rather slender. We have therefore used methods essentially similar to Graffi's to investigate the accumulation of hydrocarbons in the cells of the amphibian embryo. This material has the advantage not only that it is the tissue on which the evocatory action is exerted, but that its microsomes have been described by Brachet, while its cytoplasmic lipoids have been fully studied by Holtfreter (1946a, b, c).

Apart from the somewhat involved questions relating to Brachet's theory of evocation, a much simpler problem remains to be solved and it was hoped that the present investigations would throw some light on it. It has been apparent for some years that the cell surface plays an extremely important part in many of the morphological changes occurring during development and in particular during the formation of the neural tube (Waddington, 1942; Holtfreter, 1943, 1944b). It might with some plausibility be suggested that the carcinogenic hydrocarbons owe their evocating power to their well-known surface activity (Moricard and Gothié, 1943a, b, 1944). If this were the case, one would expect to find them localized in the surface membrane of the cell.

TECHNIQUE

Explants of the blastocoele roof of early gastrulae of the newt *Triturus alpestris* were cultured overnight in Holtfreter's standard solution containing solubilized 3:4-benzpyrene. They were then examined microscopically by ultra-violet light which causes those structures in the cell which have accumulated any of the hydrocarbon from the culture medium to show a bright blue fluorescence. The light source was an Osram high-intensity lamp with a quartz lens and filter to exclude all visible light. At first a surface-silvered mirror and quartz condenser were used on the microscope, but it was later found that the standard glass mirror and condenser gave equally good results.

Weil-Malherbe (1946) has shown that various polycyclic hydrocarbons including 3:4-benzpyrene will form water-soluble addition-compounds with caffeine and other purines. In the present investigations this method was used for rendering the benzpyrene soluble, in preference to the more complicated way of using saturated solutions in hot glycerine diluted with serum, adopted by Graffi. A 1 per cent. solution of caffeine in Holtfreter's standard solution was stirred overnight with a slight excess of benzpyrene at 20° C. and then filtered. The resulting solution, which shows distinct purplish-blue fluorescence in sunlight should, according to the figures given by Weil-Malherbe, contain about one part in 100,000 of dissolved benzpyrene. In one series of experiments this solution was diluted ten times again with Holtfreter's solution, which should give about one part in 10,000,000 of the benzpyrene. The cells of the explant cultured in this diluted solution were nearly as brightly fluorescent in ultra-violet light as were those in the stronger one and even in a solution diluted by a further factor of 10 the cells still showed a very faint bluish fluorescence. Amphibian gastrula cells cultured

in pure Holtfreter's solution or in a 1 per cent. solution of caffeine without any benzpyrene show practically no fluorescence, at the most only the very faintest greenish, not blue, tinge.

The cells of the explants in 1 per cent. caffeine in Holtfreter's solution, both with and without dissolved benzpyrene, tend to dissociate from one another and fall apart in the same way as when they are cultured in an alkaline medium. This happens also in a 1 per cent. caffeine solution buffered to pH 6.9. The dissociated cells appear otherwise to be quite healthy and remain alive for several days, although they are apparently not able to re-aggregate when transferred to a caffeine-free solution as happens when cells dissociated by alkali treatment are returned to a neutral medium (Holtfreter, 1947). Explants cultured in 0.1 per cent. caffeine with or without added benzpyrene show little or no tendency to fall apart. No cytological differences could be observed between the dissociated and undissociated cells, and, as the former in the higher concentration of benzpyrene showed a rather brighter fluorescence in ultra-violet light, they were used for most of the observations.

A few observations were made with other types of cells, particularly to search for any evidence that fluorescent compounds were accumulated in the nucleolus (see p. 215).

EXPERIMENTAL RESULTS

After having been cultured for some time in a benzpyrene-caffeine solution, the cells of the explant will have fallen apart and become spherical with a diameter of about 60μ , though there is considerable variation in size. Seen by ultra-violet light they show the characteristic blue fluorescence of benzpyrene in molecular solution, though otherwise it is hard to distinguish much structure in the spherical cell. The nucleus is seldom visible as it is obscured by the overlying fluorescent cytoplasm. When the cell is squashed slightly with a coverslip more of its structure becomes visible. The nucleus, as reported by Graffi, is entirely non-fluorescent and shows up as a dark patch in the middle of the cell. The fluorescent nucleolus described by Graffi as being occasionally visible in mouse cells was not observed in this amphibian material. Rarely a bright fluorescent liposome was seen outlined against the dark nucleus, which might have been mistaken for a nucleolus, but these were always outside the nuclear membrane.

Neither the cell membrane nor the nuclear membrane showed any fluorescence. This is true both of the intercellular membrane and of the 'surface coat' (Holtfreter, 1943, 1944a) which forms the boundary of the egg against the external medium. When a cell or group of cells is partly covered by surface coat containing pigment this material can be seen as a dark patch, itself not fluorescent, obscuring the light coming from the underlying cytoplasm. The cytoplasm showed a uniform bright blue fluorescence, though not infrequently the nucleus was outlined by a ring or crescent considerably brighter than the rest of the cytoplasm. This ring, which is usually rather homogeneous and does not contain yolk platelets, probably consists of the phospholipids

associated with the nucleus which, after fixation, constitute the so-called 'Golgi Apparatus'. It could sometimes be seen in quite fresh and unsquashed cells and was probably not an artifact. The rest of the cytoplasm in these early gastrula cells is closely packed with yolk platelets round which the lipochondria cluster in the way described and figured by Holtfreter (1946a). The lipochondria are brightly fluorescent and outline the considerably less bright yolk platelets. Scattered throughout the cytoplasm there are also a number of very much smaller brightly fluorescent granules or globules in Brownian movement as well as a few larger and very brightly fluorescent globules; both of these are considered to be liposomes. When the cells are squashed strongly so as to burst, or begin to dry up, and probably also simply as a result of prolonged exposure to ultra-violet light, they begin to show further changes. The lipochondria detach themselves from the yolk platelets and break up, their lipid constituents running together to form the larger fat globules known as liposomes which are very brightly fluorescent. As the preparation deteriorates further the liposomes become larger and less numerous as they join up with one another. At the same time as the yolk platelets lose their attached lipochondria they also lose their never very bright fluorescence.

It is probable that in an entirely fresh state the gastrula cells would have most of their lipid in lipochondria and that the liposomes appear as a result of the breakdown of the lipochondria under abnormal conditions. In cells of neurulae and later stages the liposomes do become conspicuous even in the freshest material, and they probably occur as such in the cells of the normal living embryo at those stages. In unsegmented eggs most of the lipid is in the lipochondria associated with yolk platelets although liposomes and fat globules are also present. It is, however, impossible to examine the intracellular inclusions of an unsegmented egg *in situ*; the egg must be broken up for microscopic examination of its contents and the breaking-up process is very likely to damage some lipochondria and to release their lipide as liposomes. The contents of uncleaved eggs which had been cultured in benzpyrene-caffeine solutions were examined by ultra-violet light and their yolk platelets showed the same fluorescence picture as has been described for gastrula cells. Uncleaved eggs could not, however, be cultured whole in the benzpyrene-caffeine solution since, even when the vitelline membrane is left intact, the cell membrane and 'surface coat' disintegrate after 10 minutes or so.

Some of the explants that had been cultured in benzpyrene solution were then stained unfixed with Unna's methyl green-pyronine. In this the nucleus took up the methyl green while the cytoplasmic granules of ribonucleoprotein, or microsomes, stained red with the pyronine, as described by Brachet. By this means it is easy to distinguish between the microsomes, which are rather uniform in size just at the limit of microscopic resolution and so probably about 0.3μ in diameter, and the unstained liposomes, the smallest of which were little larger than the microsomes, but which showed a graded series in size up to the largest of 1μ or more. It is hard to distinguish between them

in unstained preparations and both are small enough to show Brownian movement. By the examination of a preparation cultured in benzpyrene solution and then stained with pyronine, first of all by visible light and then immediately afterwards changing to ultra-violet, it was possible to prove that the very small granules in Brownian movement that had taken up the benzpyrene and so fluoresced in ultra-violet light were small liposomes, while the pyronine-staining microsomes showed no fluorescence. It would therefore seem that Brachet was mistaken in assuming that the ribonucleoprotein microsomes of the amphibian gastrula are able to take up benzpyrene from the surrounding culture medium (Brachet, 1947, p. 479).

As well as the microsomes, which stain a darker red, the yolk platelets and especially their attached lipochondria stain pink with pyronine. After digestion with a solution of crude pancreatic ribonuclease the pyronine-staining properties of both lipochondria and microsomes are lost, although the microsomes still remain visible, not being dissolved away.

These observations of the location of the fluorescent material within the cell were supplemented by centrifuging experiments on single uncleaved eggs and on gastrulae ground up with Kieselguhr. In both cases, the material is sorted out (in an angle centrifuge giving about 3,500 g.) into four main zones; the lipid accumulates at the centripetal pole, and is followed by a large, rather clear layer of cytoplasm, which is separated from the centrifugal layer of yolk granules by a thin zone of pigment and other granules. In material which had been treated before centrifugation with benzpyrene, the pigment layer did not fluoresce at all, while the fluorescence in the watery layer was also slight. The lipid layer was extremely bright, and there was usually fairly strong fluorescence in the yolk layer, probably due mainly to lipochondria which had not been separated from the yolk granules. The main activity was certainly in the lipid layer, as might be expected from what has been said above.

In such centrifugates, the nucleoprotein microsomes would be expected to come out at the bottom of the watery cytoplasmic layer, i.e. along with the pigment granules. No fluorescence could be found here in preparations made with a normal laboratory centrifuge. It might be argued that such an instrument would not suffice to sediment the microsomes, and that the slight luminosity of the cytoplasmic layer was due to benzpyrene absorbed on microsomes. Even if this were so, the microsomes would only account for a very small fraction of the total hydrocarbon absorbed by the cell. But the matter can be tested by further centrifugation. After a preliminary treatment in the angle centrifuge the watery layer was isolated and again centrifuged on a high-speed air-driven ultra-centrifuge of the Beams-King type. This succeeded in clearing the fluorescence; but the important point is that the granules to which the fluorescence was due moved centripetally, i.e. in the direction to be expected if they were very small fat or oil drops, but opposite to that expected if they were the microsomes discussed by Brachet.

DISCUSSION

It is clear from the above observations that the lipid constituents of the cell—lipochondria, liposomes, and perhaps the phospholipids constituting the 'Golgi Apparatus'—have an affinity for benzpyrene and will take it up out of solution with caffeine. There is really no evidence that the non-lipoid constituents can accumulate it.

The cell membrane, in particular, seems to absorb less of the hydrocarbon than any other part of the cell; in fact by the present technique none whatever can be detected in it. This finding makes it appear very improbable that the evocatory power of the steroid hydrocarbons is due to any influence of their surface activity on the membrane, although it of course remains possible that an activity of this kind plays an important role in their reaction with some deeper-lying structure within the cell.

There is also no direct observational evidence of any accumulation of benzpyrene by nucleoprotein structures. It is certainly not directly absorbed by the nucleoprotein microsomes with the avidity with which it is taken up by the lipochondria. This conclusion is not in conflict with the published evidence of Graffi. That author did, however, claim that the nucleolus, which consists largely of ribonucleoprotein, occasionally shows fluorescence with benzpyrene. It does not seem to us possible to be certain that the structures described and figured by Graffi really were nucleoli. He only rarely saw them, and it is hard to distinguish them with certainty from liposomes lying just above or below the nucleus. In the amphibian material no fluorescent nucleoli were detected, though again it would be difficult to identify them with certainty.

In order to test this matter on more favourable material a series of observations was made on the oocytes of the pond snail *Limnaea stagnalis*, which have very conspicuous nucleoli and comparatively little lipid (Raven, 1948). The ovotestis was dissected out and cultured overnight in amphibian Ringer solution diluted six times, containing 1 per cent. caffeine, and saturated either with benzpyrene or with another carcinogenic hydrocarbon, 20-methyl-cholanthrene. The oocytes were then examined either centrifuged or gently squashed under a coverslip. By visible light the nucleolus, which is about 15μ in diameter, shows up very conspicuously; on switching over to ultra-violet light a certain amount of purplish fluorescence can be seen in the yolk cytoplasm but none at all in either the nucleolus or the nucleus. Fixed oocytes of *Echinus* kept for 24 hours in the hydrocarbon solutions also showed no trace of fluorescence in their conspicuous nucleoli. Some observations were also made on the salivary chromosomes of *Drosophila* cultured in benzpyrene-caffeine solution. If ribonucleoprotein absorbs the hydrocarbon it might have been expected that the heterochromatic regions would have been fluorescent in ultra-violet light, but no sign of this could be observed. Finally, a suspension of tobacco mosaic virus (a ribonucleoprotein), stood for some hours in solubilized 3:4-benzpyrene and then centrifuged at high speed, showed no fluorescence in the centrifugate.

Weil-Malherbe (1946) detected a slight solubilizing effect on benzpyrene and other hydrocarbons by nucleotides and nucleosides *in vitro*. He suggested that this property, due presumably to their purine constituents, might be of some biological significance. It is conceivable that the ribonucleoprotein might have some slight affinity for benzpyrene but that the caffeine used as a solubilizer in this work holds on to it more strongly and so prevents its accumulation in the nucleoprotein structures. To check this point aqueous solutions of benzpyrene and methylcholanthrene were prepared according to the method described by Graffi (1939), glycerine and serum being used as the solubilizer. *Limnaea* oocytes cultured in these solutions and examined in ultra-violet light showed, however, no signs of fluorescence in the nucleolus. It must, therefore, be concluded that there is no evidence that structures containing ribonucleoproteins necessarily have any affinity for these carcinogenic hydrocarbons, whereas the evidence that the lipid constituents readily take up the substance is direct and conclusive.

The evidence that benzpyrene is not accumulated to any significant extent by nucleoprotein structures makes it probable that the mutagenic activity of the carcinogens (Demerec, 1947, 1948; Strong, 1947; Carr, 1947) is indirect, the genetic mutations being produced as secondary consequences of a primary cytoplasmic effect. It is noteworthy that, as mutagens, these substances differ in several respects from other active chemical agents, which are thought to act directly on the chromosomes, such as mustard gas (Auerbach and Robson, 1947). Thus the increase in mutation-rate achieved with carcinogens in *Drosophila* is much less than with the mustards, and although exact dosages are known in neither case, the difference is probably real. Further it has been claimed by Strong and Carr that some carcinogens (methylcholanthrene) cause the mutation of specific genes: the effect is perhaps still open to some doubt, but nothing of the kind occurs with the mustards, and the phenomenon, if true, may prove to be characteristic of secondary mutagenic action (Hadorn, 1948).

We may now turn to consider the bearing of these observations on the way in which we envisage the mechanism of action of the hydrocarbon evocators. In the first place, the survival of amphibian embryonic cells for some considerable period in the rather strong solutions used in this work makes it unlikely that the evocation is to be attributed simply to the toxicity of the substances. Holtfreter (1945) appears to have advanced this suggestion on no better grounds than that it fitted in with his theoretical outlook. He produced no good observational evidence for it; whereas Shen (1942) noted that the explants which showed the best neuralization in solutions of 1:2:5:6—dibenzanthracene- α - β -endosuccinate were the ones which had the fewest damaged and cytolyzing cells.

Sufficient attention has not always been paid to the delicacy of the balance which would have to be struck to operate the mechanism of evocation by the cytolysis of part of the exposed ectoderm. It is necessary that some cells should be actually killed, so that they release their evocator, while others

remain healthy enough to react to it by neural differentiation. It is comparatively easy to accept such an explanation in experiments where the stimulus is locally applied (e.g. by localized mechanical injury, or even by a localized implant of a relatively indiffusible chemical substance), but it is a rather less plausible explanation of the activity of an evocator which acts in solution. It then becomes necessary to suppose that some cells of the exposed ectoderm (e.g. those not protected by the 'surface coat') are much more readily accessible to the substance than the others, which are the survivors. This may in some cases be true; but the suggested mechanism has by this time become rather complicated, and there is usually no good reason to prefer it to the simpler supposition that some substances can act on the healthy ectoderm cells in such a way as to cause the release of their stores of previously inactivated evocator. If this release of the evocator occurs while the ectoderm still retains its competence then the cells would presumably respond by neuralization to the active evocatory stimulus now released within them. This is probably what happens when explants of competent ectoderm are exposed to artificial evocators in solution, where all the cells may respond by neuralization without any of them showing signs of necrosis (Shen, 1942). There is a certain similarity between an artificial evocator acting indirectly in this way and the normal evocator, since we know that when the latter diffuses from the mesoderm into the ectoderm it not only causes neuralization but also stimulates the ectoderm to produce more evocator substance, whose activity is exhibited in the phenomenon of 'homoio-genetic induction', i.e. induction of neural tissue by implants of neural tissue. We may speak of this as a *physiological activation* of the bound evocator, as opposed to the *cytolytic activation* which occurs when definitely lethal conditions, such as heat coagulation or mechanical disruption, are applied.

It is, then, very possible that the carcinogen evocators cause a physiological activation of the previously inactive evocator. If so, it would be most simple to suppose that the locus of this activation is at the lipochondria, where the hydrocarbon can be seen to accumulate. We have seen that after treatment with benzpyrene the lipochondria rather rapidly break down, their lipid constituents running together to form larger liposomes. Holtfreter (1946c) has shown that a similar process occurs in normal development and is noticeable first in the archenteron roof, that is, in the tissue in which the evocator first becomes active. It is tempting to suggest that this breakdown of the lipochondria is actually the process of liberation of free evocator from an inactive complex which was first adumbrated some dozen years ago (Waddington, Needham, and Brachet, 1936).

It should be noted that this suggestion does not entirely conflict with Brachet's hypothesis as to the importance of ribonucleoprotein granules in the induction process. Brachet (1943) has shown that such granules increase in number in the archenteron roof as it invaginates, that is, at the same time and place as the lipochondria break down. Now the lipochondria certainly consist not only of lipid but also contain protein material, probably arranged as an

envelope covering the lipide core (Holtfreter, 1946a). There is as yet no convincing evidence that this envelope is nucleoprotein in nature, but we have found that the lipochondria stain, albeit rather faintly, with pyronine and lose their affinity for that stain after digestion in crude ribonuclease. It is, therefore, by no means impossible that the appearance of Brachet's granules is not merely correlated with, but is an actual consequence of the breakdown of the lipochondria, and it is possible to suppose that they are in fact the liberated evocator.

Holtfreter (1948) drew attention to the fact that during cytolysis, when the evocator is known to become liberated, the lipochondria can be seen to break down and their lipide constituents to become free. It is clear that he considered that the correlation between the two phenomena might be significant. In his discussion of the matter, however, he does not pursue it very far; instead he turns to a mention of the cytoplasmic basiphilic granules, which he derives, not from the protein part of the lipochondria, but by an 'Entmischung' from the originally clear cytoplasm. He suggests that both the mobilization of the lipides from the lipochondria and the precipitation of granules in the cytoplasm are secondary consequences of changes in the cell surface, these being of such a kind as to lead to increased permeability. It appears to us that the demonstration that the carcinogen evocators become accumulated directly at the lipochondria, and not at the cell surface, favours the simpler hypothesis advanced above, that the evocator-liberation takes place directly at the lipochondria.

Further work is required, however, before this can be accepted as more than one of a number of possibilities. It still remains possible that it is the lipid component of the lipochondria which is the active evocator. And finally we cannot yet afford to overlook the possibility that the carcinogens are acting not as activators, either cytolytic or physiological, but as direct evocators which are chemically sufficiently allied to the natural substance to simulate its action on the ectoderm cell. What has been gained in this investigation is not a final resolution of these various alternatives, but a strong suggestion that the lipochondria are the site of evocator activation and possibly of evocator action.

SUMMARY

Cells from explants of newt gastrulae which had been cultured in Holtfreter's standard solution containing the carcinogen 3:4-benzpyrene rendered soluble by caffeine were examined for fluorescence by ultra-violet light. The lipid constituents of the cells, lipochondria and liposomes, showed the bright blue fluorescence of benzpyrene in molecular solution and there was also occasionally a structureless blue fluorescence round the nucleus corresponding perhaps to the so-called 'Golgi Apparatus'. The yolk platelets were slightly fluorescent but this was probably due to the associated lipides and it largely disappeared in moribund preparations, where the lipochondria became detached from the platelets. The nucleus showed no fluorescence, nor was a fluorescent nucleolus observed. It could be shown that the pyronine-

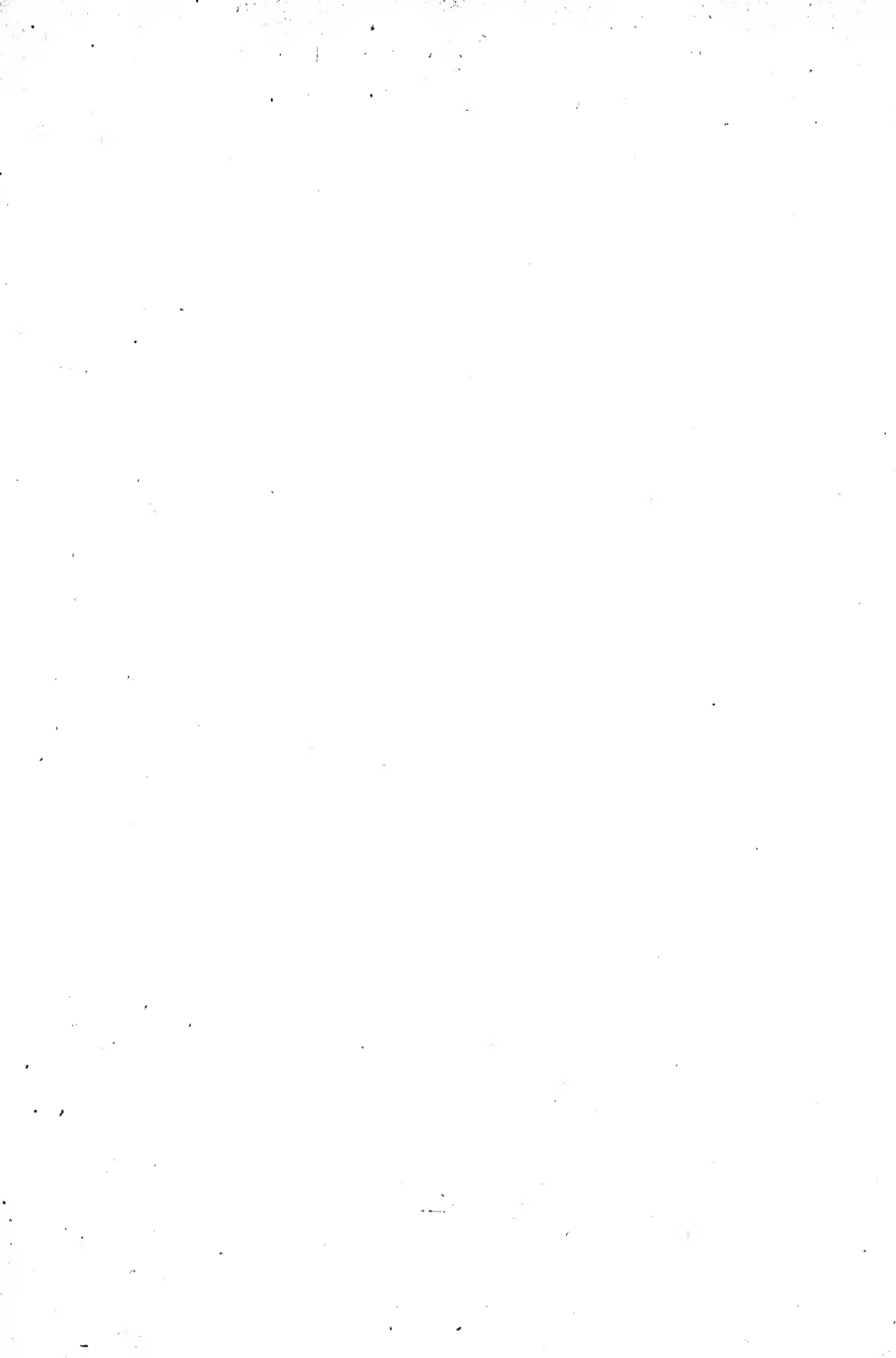
staining cytoplasmic granules of ribonucleoprotein (microsomes) were not fluorescent and so probably do not have any affinity for the carcinogen. The very small fluorescent granules in Brownian movement that had previously been looked upon as ribonucleoprotein microsomes are in fact small liposomes and do not stain with pyronine.

It is suggested that the evocating power of the carcinogenic hydrocarbons is probably not due to *cytolytic activation* of the evocator, by the killing of some cells in the exposed ectoderm. It may be by *physiological activation*, operating through a specific action on the lipochondria, leading to their breakdown into liposomes and the release of active evocator substance. Some evidence is produced which suggests that the lipochondria contain ribonucleoprotein, and this fraction of them may constitute the active evocator; this would be congruent with the theories of Brachet (1947). It cannot yet be excluded, however, that the hydrocarbons act as *direct evocators*, simulating the effects of the naturally occurring substance.

The observation that benzpyrene is not accumulated by nucleoprotein makes it likely that the mutagenic activity of the carcinogens is indirect.

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Observations on the Branchial Crown of the Serpulidae (Annelida, Polychaeta)

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INTRODUCTION

DURING investigations on the blood systems of serpulids (Hanson, 1949) observations were made on the internal anatomy of the branchial crown. Previous accounts were found to be incomplete and in some respects inaccurate; so I decided to publish some of my observations. Thomas (1940) has given the most recent general description of the crown of a serpulid, *Pomatoceros*. Pruvot (1885) and Johansson (1927) showed, by studies on its innervation, that the crown of the Serpulimorpha represents the two prostomial palps of other polychaetes. It consists of filaments bearing pinnules, of the so-called 'palps', and, in most serpulids, of an operculum borne on a peduncle. It is generally recognized that the operculum and peduncle represent a modified filament (Zeleny, 1905; Segrove, 1941).

The following species were used: *Serpula vermicularis* L., *Hydroides norvegica* (Gunnerus), *Vermiliopsis infundibulum* (Philippi), *Pomatoceros triqueter* L., *Salmacina incrustans* Claparède, *Protula intestinum* (Lamarck), *Spirorbis corrugatus* (Montagu), and *S. militaris* (Claparède). *Pomatoceros* was obtained from Plymouth. The other species were studied at Naples. Most of the observations were made on sectioned specimens. The fixing solutions and staining methods I used are listed in my account of the blood system in the Serpulimorpha (Hanson, 1949).

OBSERVATIONS

Muscles

The muscles of the filaments (Text-fig. 1), pinnules (Text-fig. 6), 'palps'

(Text-fig. 7), and opercular peduncle (Text-fig. 2) of *Pomatoceros* are all longitudinal.

In each filament are the following muscles:

(a) Two external branchial muscles (Thomas, 1940) situated in the two corners of the abfrontal side of the filament. Near to them are the two external branchial nerves.

(b) Two internal branchial muscles (Thomas) situated on the frontal side of the filament. The fibres of these muscles are grouped round the internal branchial nerve. Most of them lie on each side of the nerve, but some are abfrontal to it.

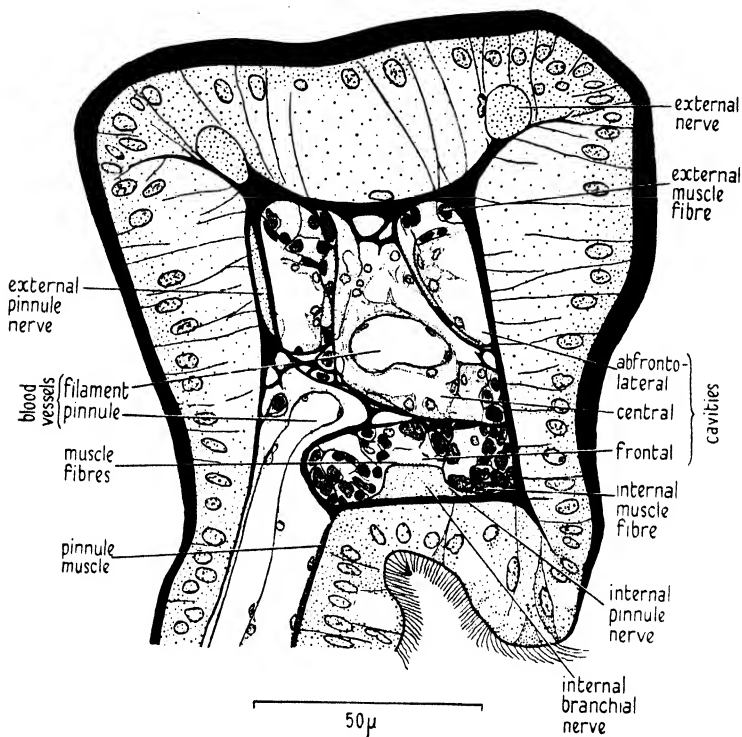
(c) A single layer of small muscle-fibres situated on the abfrontal surface of the internal branchial nerve. They are not present on the external branchial nerves or on any other nerves except the internal peduncular nerve.

The peduncle (Text-fig. 2) is triangular in cross-section, and contains a single group of muscle-fibres situated in the apical angle (frontal) and along the two sides. The fibres do not enter the operculum, but terminate at the top of the peduncle. Here they are attached to the basement membrane of the epidermis which roofs the peduncle, except for a small aperture through which the blood-vessel and its surrounding connective tissue and the three opercular nerves enter the operculum. McIntosh (1922-3) stated that the muscles of the peduncle are 'chiefly transverse', and concluded: 'The thrusting out of the operculum—if such happens—is thus voluntary, the withdrawal and retention more or less involuntary.' I have found that the peduncular muscle, like the branchial muscles, consists of longitudinal fibres, the contraction of which will draw the operculum towards the centre of the crown when the animal withdraws into its tube. When the crown is expanded the peduncle is sometimes held out nearly at right angles to the long axis of the body.

Each pinnule (Text-fig. 6) and 'palp' (Text-fig. 7) has only a few muscle-fibres, situated just inside the basement membrane of the frontal epidermis.

A pair of internal and a pair of external branchial muscles are present in each filament of *Serpula* (Text-fig. 3), *Hydroides*, *Vermiliopsis*, *Salmacina*, and *Spirorbis militaris*. As in *Pomatoceros*, the external branchial muscles are less well developed than the internal branchial muscles, but they are more reduced in these serpulids than in *Pomatoceros*. In *Protula* and *Spirorbis corrugatus* external branchial muscles could not be found, but internal branchial muscles are present. In their internal structure the peduncles of both functional and reserve opercula of *Serpula* and *Hydroides* are very similar to filaments; the internal and external peduncular muscles have the same appearance as their counterparts in the filaments. The arrangement of the muscles in the pinnules and 'palps' of *Serpula*, *Hydroides*, and *Vermiliopsis*, and in the pinnules of *Protula* and *Spirorbis militaris* is the same as in *Pomatoceros*. Muscle-fibres on the abfrontal surface of the internal branchial nerve have been found in the following serpulids besides *Pomatoceros*: *Serpula*, *Hydroides*, *Vermiliopsis*, *Protula*, and *Spirorbis militaris*. Their function is not known. Comparable

muscles of unknown function occur on the dorsal surface of the ventral nerve cord of *Nereis* and other errant polychaetes (Prenant, 1929), and on the lateral nerves of pelagic nemerteans (Coe, 1926). Probably other cases exist.



TEXT-FIG. 1. Transverse section through filament of *Pomatoceros triqueter*, and longitudinal section through base of one pinnule.

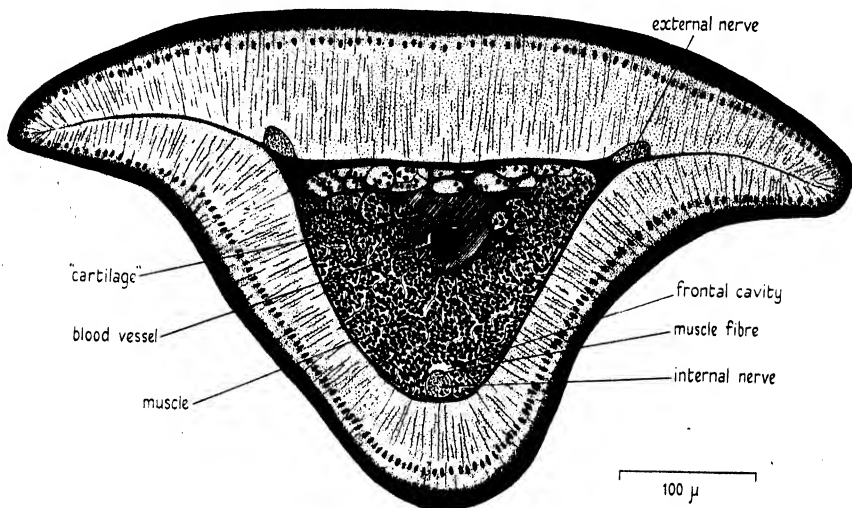
Nerves

In the *peduncle* and each *filament* of *Pomatoceros* there are three nerves (Thomas, 1940). Two are situated in the abfrontal corners (the external peduncular and external branchial nerves) and the third on the frontal face (internal peduncular and internal branchial nerves). The abfrontal nerves lie outside the basement membrane of the epidermis. The frontal nerve lies just inside the basement membrane. On its abfrontal surface is a layer of small muscle-fibres (see above) and a small well-defined canal, part of the system of cavities in the crown (p. 229). No muscles or canals accompany the abfrontal nerves.

All three branchial nerves send branches into the *pinnules*. The branches of the internal and external branchial nerves are respectively the frontal and abfrontal nerves of the pinnules (Text-figs. 1 and 6). The nerves of the

pinnules lie outside the basement membrane of the epidermis. Thomas found the frontal pinnule nerves, but has stated that abfrontal nerves are absent.

At the top of the *peduncle* the muscles and canal accompanying the internal peduncular nerve terminate, and the two external peduncular nerves come to lie just inside the basement membrane of the epidermis. Each of the three nerves now gives off two small branches which I have been able to trace for a short distance only. The three nerves enter the operculum through the



TEXT-FIG. 2. Transverse section through opercular peduncle of *Pomatoceros triqueter*.

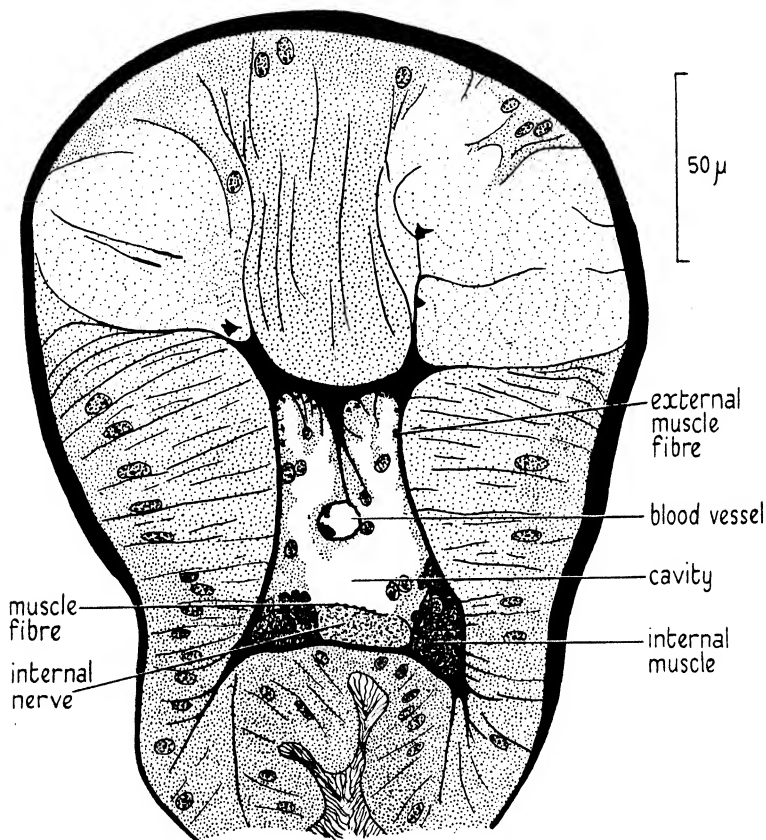
small gap by which the inside of the operculum communicates with the inside of the peduncle. Within the operculum all three nerves pass to the outside of the basement membrane. They give off many small branches, and can be traced as far as the rim round the top of the operculum.

The internal and external branchial nerves of the following serpulids are like those of *Pomatoceros*: *Hydroides*, *Vermiliopsis*, *Protula*, *Salmacina*, *Spirorbis corrugatus*, and *S. militaris*. No external branchial nerves could be found in *Serpula*. All my material was stained with 'Azan', however; different histological methods might reveal them, although they are distinct in Azan-stained sections of other serpulids. The internal and external peduncular nerves of *Hydroides* are like the corresponding nerves in the filaments. Both internal and external pinnule nerves have been found in *Hydroides*; internal pinnule nerves are present in *Vermiliopsis*, *Protula*, and *Spirorbis militaris*, but external pinnule nerves could not be found. Meyer (1888) was unable to find external pinnule nerves in *Eupomatus lunuliferus* (= *Hydroides lunulifera*) or *Psygmobranchus protensus* (= *Protula tubularia*), but Faulkner (1930) saw them in *Filograna implexa*.

The functional significance of the double nerve-supply to the serpulid crown is not known.

Skeleton

The internal skeleton of the filament of *Pomatoceros* consists of fibres enmeshing the muscle-fibres of the internal and external branchial muscles,



TEXT-FIG. 3. Transverse section through filament of *Serpula vermicularis*.

and of sheets, namely, the basement membrane of the epidermis and the skeletal coat of the blood-vessel wall (Hanson, 1949). Both the fibres and the sheets give the staining reactions of collagen. The fibres, and the basement membrane of the epidermis, but not the skeletal coat of the vessel wall, enclose reticular fibres. No elastic fibres are present.

In the *peduncle*, as in the filaments, the muscle-fibres are enmeshed by connective tissue-fibres. On the abfrontal surface of the peduncular muscle these fibres pass into the ground substance of a cartilage-like tissue which

occupies the abfrontal part of the peduncle and fills the operculum, except for the space taken by the spiral opercular blood-vessel (Hanson, 1949). The ground substance gives the staining reactions of collagen, and is penetrated by reticular fibres and by a network of cytoplasm containing nuclei (Text-fig. 4). Ewer and Hanson (1945) have found that the ground substance reacts negatively to mucicarmine, thionin, resorcin-fuchsin, orcein, and safranin, all of which stain the ground substance (mucoprotein) of mammalian cartilage in a characteristic ('positive') manner. The cartilage-like tissue of *Pomatoceros* is very similar to the 'perichondrial' part of the branchial skeleton



TEXT-FIG. 4. Photomicrograph of section through 'cartilage' in operculum of *Pomatoceros triqueter*. Flemming. Iron haematoxylin.

of *Sabella pavonina*, as described by Nicol (1930). Ewer and Hanson have found that the 'perichondrium' of *S. spallanzanii* reacts negatively to mucoprotein stains, but that the thick walls of the large vacuolated cells which make up the axial part of the skeleton are coloured by these stains in the same manner as mammalian cartilage (and sabellid mucous cells); they presumably contain a mucoprotein. Some similar results were obtained by Nowikoff (1912).

In the *pinnule* and 'palp' of *Pomatoceros* and other serpulids, the internal skeleton is represented only by the basement membrane of the epidermis and the skeletal coat of the vessel wall.

In the filaments of serpulids other than *Pomatoceros*, external branchial muscles either appear to be absent or are represented only by a few fibres lying near the basement membrane of the abfrontal epidermis (Text-fig. 3). There are no connective tissue-fibres enmeshing these muscle-fibres. The skeleton associated with the internal branchial muscles is as well developed as in *Pomatoceros*. In *Hydroides*, a single stout fibrous sheet stretches from the skeletal coat of the blood-vessel wall to the medial line of the basement membrane of the abfrontal epidermis (figured by McIntosh, 1926). *Serpula*

(Text-fig. 3) and *Protula* are similar. In *Vermiliopsis* the vessel is anchored by several such fibrous sheets. In *Salmacina*, *Spirorbis militaris*, and *S. corrugatus* the vessel lies on the basement membrane of the abfrontal epidermis. In *Pomatoceros* the vessel lies in the centre of the filament, and fine fibres anchor it mainly to the skeleton around the external and internal branchial muscles.

The branchial skeleton of *Sabella* consists of large, vacuolated thick-walled cells surrounded by a cartilage-like sheath (Nicol, 1930). I have seen a similar skeleton in *Potamilla* sp. and *Dasychone lucullana* Delle Chiaje. No serpulid has yet been found to possess a skeleton like this. Large vacuolated cells with strong walls are commonly found in the epidermis of the serpulid crown, e.g. in *Filograna implexa* (Faulkner, 1930) and *Protula* (= *Salmacina*) *dysteri* (Huxley, 1855). In *Pomatoceros* their walls are not as thick as those of the axial part of the sabellid skeleton, and do not give the same staining reactions.

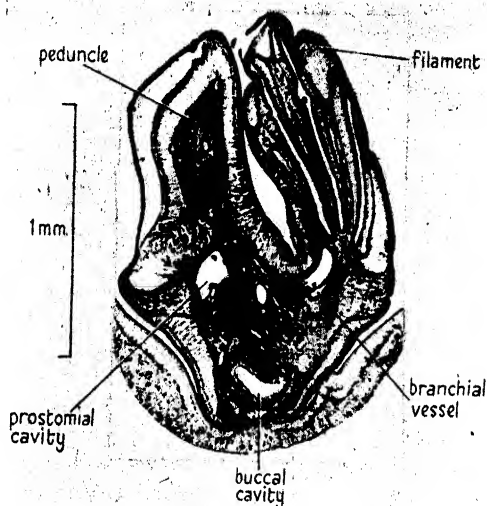
The internal skeleton in the peduncles of the functional and reserve opercula of *Serpula* and *Hydroides* is very similar to that in their filaments. 'Cartilage', of the same type as in *Pomatoceros*, is confined to the opercula which it fills, except for the spaces taken by the branched blood-vessels. The 'cartilage' in the reserve opercula has a much denser cell population than in the functional opercula. The 'cartilage' of *Serpula* and *Hydroides* was described by Örley (1884) and Zeleny (1905), respectively.

Cavities

The cavities in the prostomium and peristomium of *Pomatoceros* have processes extending into the base of the crown, where they end blindly. I have found that they are not, as previously stated (McIntosh, 1918; Thomas, 1940), continuous with the cavities in the filaments. The nature of all these cavities is obscure. 'It is difficult to determine whether the cavity in this region, which represents the head and collar segment, is of blastocoelic or coelomic origin . . .' (Segrove, 1941). Observations now to be reported have been made on the arrangement of the cavities in the peristomium, prostomium, and base of crown of *Pomatoceros*, and in the filaments, pinnules, 'palps', and peduncle of *Pomatoceros* and other serpulids.

The posterior septum of the cavity of the peristomium of *Pomatoceros* joins the alimentary canal at the junction between the oesophagus and the stomach. The main part of the peristomial cavity extends forwards as far as the level of the cerebral ganglia, and two processes from it accompany the two branchial blood-vessels to the base of the crown. I have found that the ventral mesentery extends throughout the peristomium as far as its front wall, and does not end behind the peristomium as stated by Thomas (1940). The peristomial part of the dorsal mesentery extends forwards only as far as the level of the transverse canal joining the two thoracic nephridia. Here the tissue of the dorsal mesentery becomes detached both from the roof of the peristomial cavity and from the wall of the oesophagus, and becomes a sheath around the dorsal blood-vessel. Posteriorly, the peristomial cavity consists of two halves,

right and left, separated by the dorsal and ventral mesenteries. Anteriorly it is irregularly subdivided by the ventral mesentery and by strands of tissue stretching from the body-wall to the wall of the oesophagus. The ciliated funnels of the two thoracic nephridia open laterally into the peristomial cavity in this anterior region. If, as Meyer's work on *Protula tubularia* (Meyer, 1888) seems to indicate, these ciliated funnels are coelomoducts, the fact that they open into the peristomial cavity suggests that this cavity is



TEXT-FIG. 5. Photomicrograph of obliquely transverse section through base of crown of *Pomatoceros triqueter*.

coelomic, and the presence of dorsal and ventral mesenteries strengthens this supposition.

The cavity of the prostomium is confined to the dorsal part of the animal; it extends posteriorly for a short distance behind the cerebral ganglia, and anteriorly into the base of the crown. It is separated into two halves, right and left, by a septum enclosing the common median duct of the nephridia.

The base of each branchial blood-vessel lies in a process of the peristomial cavity, passing forwards between the dorsal and ventral roots of the circum-oesophageal nerve commissures, and ending blindly before the branchial vessel gives off the first of the filament vessels. Also accompanying each branchial vessel is one-half of the cavity of the prostomium (Text-fig. 5); it extends round the base of the crown and ends blindly where the branchial vessel enters the most ventral filament. The cavities of the prostomium and peristomium do not communicate with each other. On the left side each sends a blind-ending branch into the base of the peduncle; they accompany the

opercular blood-vessel, which is a branch of the left branchial vessel. The peristomial and prostomial cavities have no connexion with the cavities in the filaments.

McIntosh (1918) noticed cavities in the filaments of *Pomatoceros*, and thought it probable that they communicate with the 'coelomic' space around the branchial vessel, although he was not able to find the communication. He did not describe the derivation of this 'coelomic' space, but from his figures it is clear that he was referring to the anterior part of the prostomial cavity. Thomas (1940) has stated that the 'coelom' of the peristomium 'sends a pair of extensions passing between the dorsal and ventral roots of the oesophageal connectives giving branches into the filaments . . .'. Her figure of a transverse section through one of the filaments (Fig. 8) suggests that she has mistaken as a coelomic channel the space which usually appears in fixed specimens between the wall of the vessel and the mass of coagulated blood lying in its centre. It may be concluded that she has not demonstrated any extension of the peristomial cavity into the filaments. Meyer (1888) thought that the 'secundäre Leibeshöhle des Kopfmundsegmentes' of *Hydroides lunulifera* and *Protula tubularia* is continuous with the channel surrounding the vessel in the centre of each filament. Faulkner (1930) has stated that in *Filograna implexa* the prostomial cavity sends a process on each side into the base of the crown, with branches into each of the filaments.

Each *pinnule* and '*palp*' of *Pomatoceros* contains a large central cavity lined by a thin epithelium lying on the basement membrane of the epidermis. In the frontal part of the lining are situated the longitudinal muscles; fused to the abfrontal lining is the blood-vessel.

The cavities of the pinnules are branches of the central cavities of the filaments (Text-fig. 1). Each filament contains four cavities, which extend without interruption along the whole of its length.

(a) A central channel surrounding the blood-vessel.

(b, c) A pair of abfronto-lateral channels situated between the lateral epidermis and the external branchial muscles. These are probably the 'coelomic' cavities of McIntosh (1918).

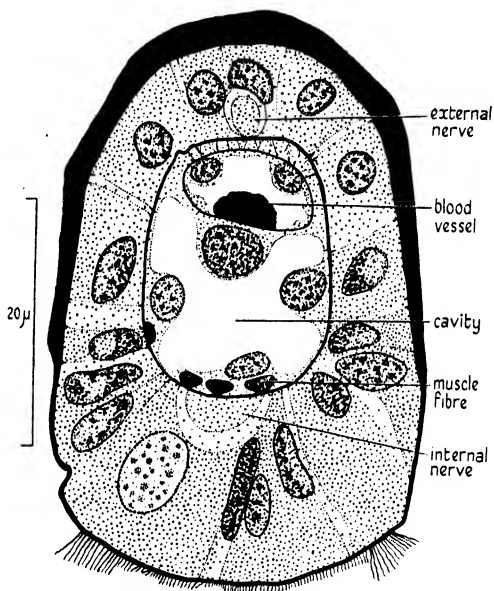
(d) A small channel situated on the abfrontal face of the internal branchial nerve, and separated from the central cavity by some of the fibres of the internal branchial muscles and their surrounding connective tissue.

The *operculum* contains no cavities. The first three cavities of the filaments are not represented in the *peduncle*, but the internal peduncular nerve is accompanied by a small channel like that of the internal branchial nerve (Text-fig. 2).

In the following serpulids the pinnule cavity is like that of *Pomatoceros*, but in the filaments there is only a single cavity, which is large and occupies most of the interior: *Serpula* (Text-fig. 3), *Hydroides*, *Vermiliopsis*, *Protula*, and *Spirorbis militaris*. The cavities in the peduncles of the functional and reserve opercula of *Serpula* and *Hydroides* are like filament cavities.

The 'palps'

The so-called 'palps' of *Pomatoceros* are two short processes situated between the dorsal lip and the most dorsal filaments of the two halves of the crown. Studies on the feeding method (Johansson, 1927; Thomas, 1940) have shown that the 'palps' are concerned with the rejection of unwanted particles. Except in a few minor points, the structure of a 'palp' of *Pomatoceros* is identical with that of a pinnule.



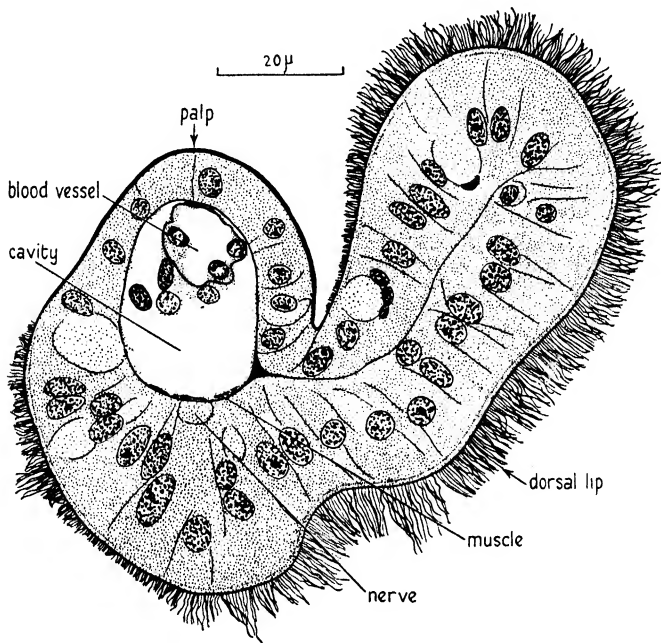
TEXT-FIG. 6. Transverse section through pinnule of *Pomatoceros triqueter*.

At its base the 'palp' is fused both with the adjacent filament and with the dorsal lip. A short distance from the base it becomes detached from the filament, but remains attached to the lip, from which it becomes free only at its tip. The lip consists of two epithelia, whose basement membranes are fused except where blood-vessels and nerves are situated between them. As Thomas has shown, these nerves arise from a branch of the internal branchial nerve of the most dorsal filament of each side. The lip vessels have no connexion with the 'palp' vessels, and are supplied with blood from the circum-oesophageal vessels (Hanson, 1949).

The epidermis of the 'palp' (Text-fig. 7) rests on a basement membrane inside which is the central cavity, lined by a thin epithelium as in a pinnule. The disposition of the muscles and blood-vessel of the 'palp' is the same as in a pinnule. The vessel is a branch of the vessel of the adjacent filament. As Thomas has shown, the internal branchial nerve of this filament gives off a

branch which innervates both the 'palp' and the dorsal lip. In the 'palp' the nerve is situated at the base of the frontal epidermis; it corresponds to the internal pinnule nerve. I have been unable to find an abfrontal nerve in the 'palp', although one is present in the pinnule.

The very close structural resemblance between 'palp' and pinnule suggests that the 'palp' may be a modified pinnule (compare Text-figs. 6 and 7). This



TEXT-FIG. 7. Transverse section through 'palp' and accompanying part of dorsal lip of *Pomatoceros triqueter*.

is supported by the fact that its blood-vessel and nerve are branches of those of the adjacent (most dorsal) filament. Moreover, the arrangement of pinnules at the base of this dorsal filament is atypical. In other filaments the pinnules are arranged in pairs from the base to the tip. In each of the most dorsal filaments there are two unpaired pinnules situated below the most basal paired pinnules, and both arise from the side adjacent to the 'palp'. Segrove (1941) has noticed that, at an early stage in development, the basal pinnule of the most dorsal filament 'is unpaired and arises on the median side of the filament'.

Segrove's account of the early development of the branchial crown, however, does not lend any support to the view that the 'palp' is a modified pinnule. He has found that the crown first appears as six primary branchial processes, three on each side of the head. The most dorsal pair develops into the 'palps',

and the others into filaments, except one on the left side which becomes the operculum and its peduncle. At first, the ciliation of the primary branchial processes is like that of adult pinnules. The 'palps' retain this type of ciliation, do not grow as rapidly as the other processes, and do not develop pinnules. The other processes acquire the ciliation of filaments when their pinnules develop. This account of the external appearance of the developing crown suggests that the 'palps' represent filaments which have retained their juvenile ciliation. However, it is possible that they are precociously developed pinnules belonging to the adjacent filaments. It is clear that information about the internal anatomy of the crown of very young specimens is needed to elucidate the morphological nature of the 'palps'. The development of the 'palps' of the sabellid *Branchiomma* (Wilson, 1936) is similar to that of *Pomatoceros*.

The 'palps' of *Serpula*, *Hydroides*, and *Vermiliopsis* are like pinnules in structure, and their blood-vessels are branches of the vessels supplying the most dorsal filaments in the crown.

Part of this investigation was made at the Zoological Station of Naples. I am grateful to the staff of the Station, to the British Association for the Advancement of Science for the use of its Table, and to the University of London for a grant towards travelling expenses.

SUMMARY

1. This paper records various observations supplementing, and in some cases correcting previous accounts of the internal structure of the branchial crown in *Pomatoceros triqueter*, *Serpula vermicularis*, *Hydroides norvegica*, *Vermiliopsis infundibulum*, *Salmacina incrustans*, *Protula intestinum*, *Spirorbis corrugatus*, and *Spirorbis militaris*.

2. The muscles in the opercular peduncle of *Pomatoceros* are longitudinal.

3. A single layer of small longitudinal muscle-fibres, of unknown function, has been found on the abfrontal face of the internal branchial and internal peduncular nerves of most of these serpulids.

4. External branchial muscles are well developed in *Pomatoceros*, reduced in *Serpula*, *Hydroides*, *Vermiliopsis*, and *Spirorbis militaris*, and apparently absent in *Protula* and *Spirorbis corrugatus*.

5. The pinnules of *Pomatoceros* and *Hydroides*, like filaments and opercular peduncles, have a double innervation.

6. The internal skeleton of the serpulid crown consists of sheets and strands of connective tissue-fibres and, in the opercula, of a cartilage-like tissue with a ground substance giving the staining reactions of collagen. The serpulid branchial skeleton is compared with that of sabellids, and the chemical nature of these skeletons is discussed.

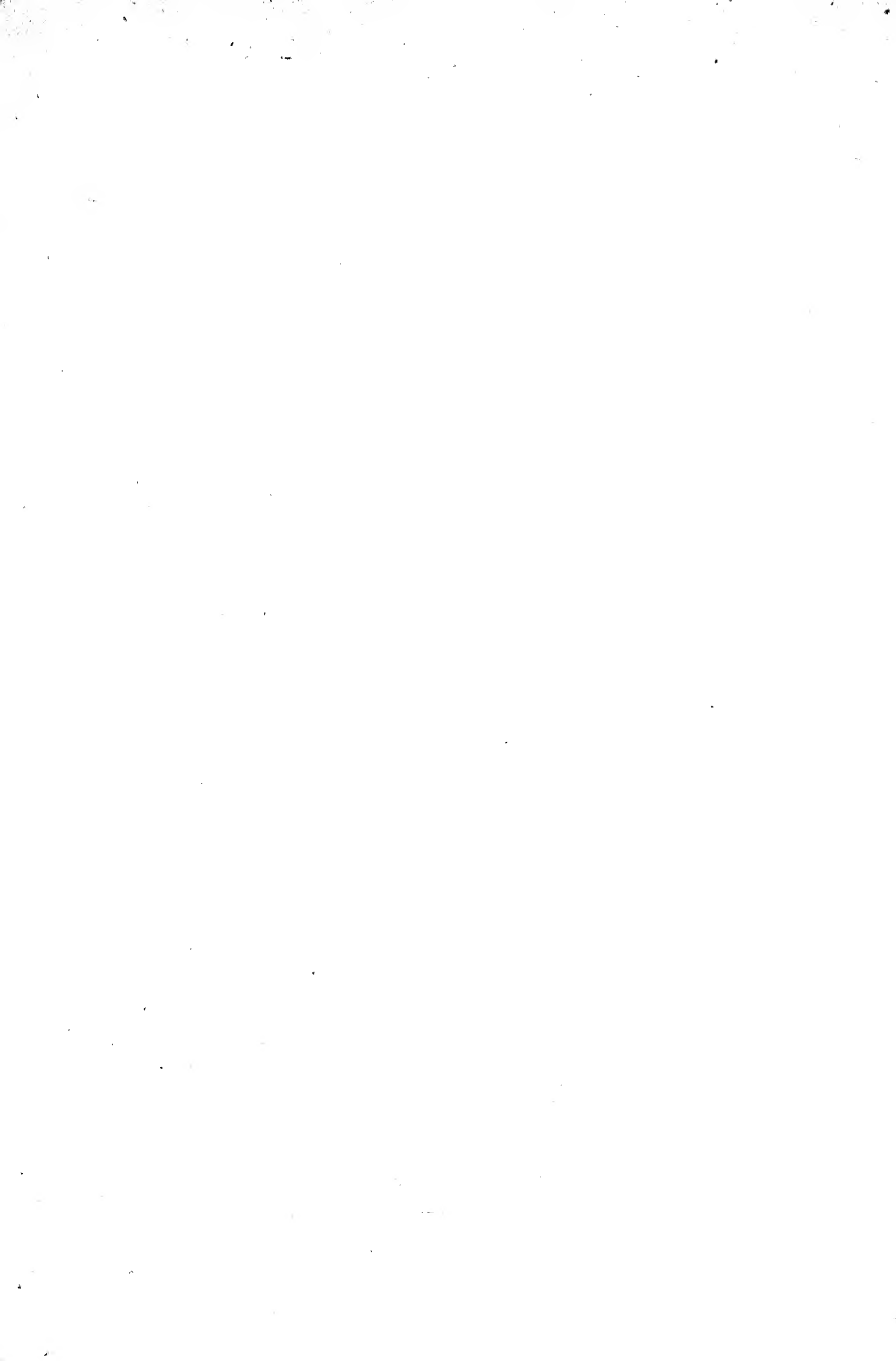
7. Extensions of the prostomial and peristomial cavities accompany the branchial blood-vessels into the base of the crown, but have no connexion with the cavities in the branches of the crown. The arrangement of the latter

is described. The nature of all these cavities, whether coelomic or blastocoelic, is obscure.

8. The 'palps' of *Pomatoceros*, *Serpula*, *Hydroides*, and *Vermiliopsis* closely resemble pinnules. The blood-vessel and nerve of the 'palp' of *Pomatoceros* are branches of those in the adjacent filament. It is suggested that the serpulid 'palp' is a modified pinnule.

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The Polymorphic Transformations of *Obelia*

BY

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Part I. Stolonie growth and Perisarc

Part II. Hydranth development

Part III. Gonangium and Medusae

INTRODUCTION

THE following account is a portrayal of a single hydroid species, *Obelia commissularis*, as a living organism in all of its manifestations.

Obelia has long been established as the text-book type of a marine hydroid, illustrating the colonial condition and so-called alternation of generations. In the present century it has been the subject of several special studies. Huxley and de Beer (1923) have given a detailed account of the process of hydranth resorption. Lund (1921-6), in a series of papers, correlated hydranth formation and regeneration with electrical polarities. Commencing in 1933 and culminating in a general review started in 1943, Hammett and his co-workers published numerous papers on the role of amino-acids and other factors upon the several phases of growth as defined by them. *Obelia geniculata* was employed as the test organism, and while the approach is dynamic and numerous observations on the formation of the living hydranth are reported (Hammett, 1943; Hauscha, 1944), the 'concern here is not with organism as organism; but with organism as test material suitable for evaluation of the interdependencies of growth'. Much of the value of this work depends upon the validity of the various abstractions made of the developmental process as a whole. Since our own emphasis is on *Obelia* as an organism, it is unnecessary to discuss the question further at this time. In later papers Hammett (1945-6) presents statistical analyses of the condition of the hydranth population of thousands of colonies, undoubtedly a herculean labour but one that throws into sharp relief the difficulty, if not the impossibility, of analysing individual behaviour in terms of statistical averages, for an organism persists in being primarily organismal in spite of anything an investigator may have in mind.

Studies of *Obelia* are included in the older work of Weismann, in his monograph (1883) on the origin of the sex cells in hydroids. Weismann's illustrations are unequalled in beauty and accuracy, but they were interpreted in an extremely static manner remote from any concept of a living organism,

¹ Aided by a grant from the Penrose Fund.

and with a misleading preconception of the continuity and nature of germ plasm, a theory ostensibly but not actually based upon these particular studies (cf. Berrill and Liu, 1948).

The present account was completed before acquaintance was made with the work of Louis Agassiz of almost a century ago. Yet it was Agassiz (1862), with his profound belief in special creation and his idea of an organism as being as final 'as an expressed thought', who saw *Obelia* and other hydroids simply as they are. With the aid of A. J. Clark he described and pictured the hydroids in living detail that has not since been equalled. Our own account, even though independent, may well be regarded as a direct extension of Agassiz's observations, both in kind and in motive, being essentially a deep interest in and respect for the organism as such.

THE MATURE COLONY

Obelia commissuralis McCrady is a species common along the New England coast, where it forms large branching colonies that attain a length of 20 cm. or more. Mature colonies consist usually of a few long stems bearing secondary and tertiary branches, and united proximally to form a narrow base of attachment. The extent and habit of growth sharply distinguishes it from *O. geniculata* and other species, *O. longissimus* being the only one with which it might be confused. The form of growth is related to the presence of long distal processes, with few branches and no hydranths, which are in fact large free terminal stolons. These are found at all seasons of the year and are not purely a response to high summer temperatures as in the case of *Bougainvillia superciliaris* (cf. Berrill, 1949). They give a distinctive appearance to the colony. The main lateral branches also end in stolons, though narrower and not so long.

Below the distal stolon zone the hydranths are formed profusely. Gonangia develop only in the proximal regions of the colony, forming from the angles of hydranths and lateral branches. Annulations of the perisarc, usually three or four, separate the internodes and occur also at the base and tip of each hydranth stalk.

The questions presented by the general form and detail of a colony are the nature of stolons, wherever they appear, the development of hydranths, gonangia, and medusae, and the relationship between them; and the origin and significance of annulations, together with the formation and influence of chitin.

GROWTH CYCLE

The preceding description presents a somewhat static picture. In reality there is a continual change in all parts of a colony, and the organism which is *Obelia* can be properly defined only as activity. This is sharply brought out by the behaviour of isolated pieces of distal stolon, especially when allowed to attach to glass at a temperature of about 15° C. Under these circumstances a cyclic transformation takes place.

The distal end continues to grow forward, though more slowly. The proximal end becomes progressively attenuated as cells degenerate and withdraw into the hydropasm. The effect is a migration of the stolon piece as a whole, much like the motion of an amoeba in spite of multicellularity and slowness. No annulations appear in the attached stolon.

A short distance behind the advancing tip a vertical branch appears. This is annulated from the first, and at its distal end it develops into a hydranth. At the same time the stolon tip continues to advance and another and similar upgrowth arises a little behind the tip. The process goes on until such vertical branches with terminal hydranths stand more or less equidistantly down the whole length of the stolon. In the proximal half, however, there is not only a slow resorption of the basal attached stolon but resorption of formed hydranths.

The overall picture of the cycle is shown in Text-fig. 1C. Behind the advancing tip of such newly formed stolons, in orderly succession, there is usually a short annulated vertical branch, a longer growth with a developing terminal bud, one or more such branches with functional hydranths, a hydranth in process of resorption, and finally several perisarcal tubes containing coenosarc as attenuated as the basal stolon of this region. So it continues, new stolon growth and new hydranths forming distally, resorption of the old occurring proximally, the resorbed material passing into the hydropasmic stream and serving as nutrient for the building of the new.

PART I. STOLONIC GROWTH AND PERISARC

STOLONIC GROWTH

Stolon growth occurs at the free end of the main and lateral branches as part of the normal growth of this species. It also occurs to a more limited extent as basal attached stolons, and also from either end of cut pieces of stem. The process appears to be the same wherever it appears, except for variation in diameter, growth-rate, and the effect of contact.

RATE OF GROWTH

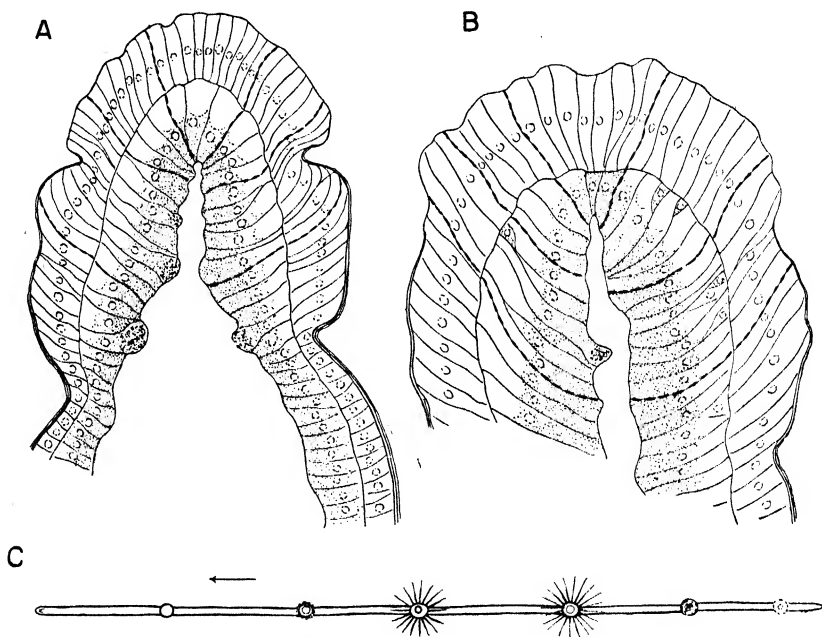
With optimum nutritive conditions, i.e. maintained either by actively feeding or by actively resorbing hydranths, the rate of stolon growth varies directly with the temperature. Under such conditions the rate of growth at 10–12° C. is a little less than 1 mm. in 24 hours; at 16–17° C. it is about 10 mm. in 24 hours; and at 20° C. it may be as much as 15–20 mm. in 24 hours. Rapid growth continues at temperatures as high as 25° C., possibly 26° C., but at 27° C. growth ceases even though the tissue may appear to be otherwise healthy.

In most cases the maximum growth-rate possible for a given temperature is maintained only for limited periods, for production of developing hydranths behind the stolon tip introduces competitive demands upon the nutritive supply. Ideally, however, stolon formation is a process of rapid continuing

growth with no recognizable decrement such as is characteristic of a developing hydranth or other organized structure.

PROCESS OF GROWTH

A detailed study of stolon tips reveals significant cell arrangements. Whether a stolon is massive or slender, the cells of both the epidermis and



TEXT-FIG. 1. A, B. Sections through two stolon tips, respectively growing slowly and rapidly, showing confluence of cell contours of epidermis and endodermis (emphasized by heavy broken lines), and also showing graded vacuolation of distal endodermal cells. Note absence of vacuolation in the terminal cells of the more rapidly growing stolon (B). C. Diagram of growth sequence of isolated stolon, new stolon tissue forming distally and old resorbing proximally, direction of growth indicated by arrow. In sequence along stolon are initial and late stage of developing hydranth, two mature hydranths, and hydrothecae of two resorbed hydranths.

endodermis are streamlined as though flowing in and constituting a single current moving toward the tip. The intercellular contours of the endodermis continue with those of the epidermis. Whatever force is responsible for the configuration, the appearance is that the cells of both layers flow in conformity with the lines of force and are subordinate rather than determinate (Text-fig. 1A, B).

Cell proliferation has been found only at the extreme tip of both epidermis and endodermis. In neither layer is there any sign of so-called interstitial cells and in any case they must be so rare that no significant role can be

assigned to them. Since stolon growth is linear and in one direction, proliferation must in a sense be polarized, that is, the cells formed toward the proximal side of the proliferation zone cease division, those on the distal side continue to divide.

Immediately behind the proliferative tip the endodermal cells present a very distinctive appearance. Toward the hydrocoel or lumen they retain their characteristic granular nature, but on the side toward the epidermis the cells become extended with a large clear vacuole. Passing backwards from the tip, vacuolization rises rapidly to a maximum and then slowly decreases, finally disappearing altogether (Text-fig. 2B), when the cells become indistinguishable from those of the endodermis in general. When growth is very rapid the terminal proliferating endodermal cells may be granular throughout and somewhat smaller than the vacuolate cells. When growth is slower, the terminal cells themselves may be vacuolate.

The cell cycles appear to be as follows. In the terminal division centre of the endodermis the cells may be relatively small if division is very rapid, but if not so rapid, and in any case at the margin of the centre, the cells become greatly enlarged and vacuolated, the vacuolated cells probably dividing at least once. The vacuolation is probably a polar imbibition of water, is definitely a temporary phase, and is in some way related to the rejuvenescence or reconstitution of cell organization. The phenomenon is seen elsewhere in the activated epicardial tissue of the ascidian *Archidistoma* at the time of strobilation (Berrill, 1948).

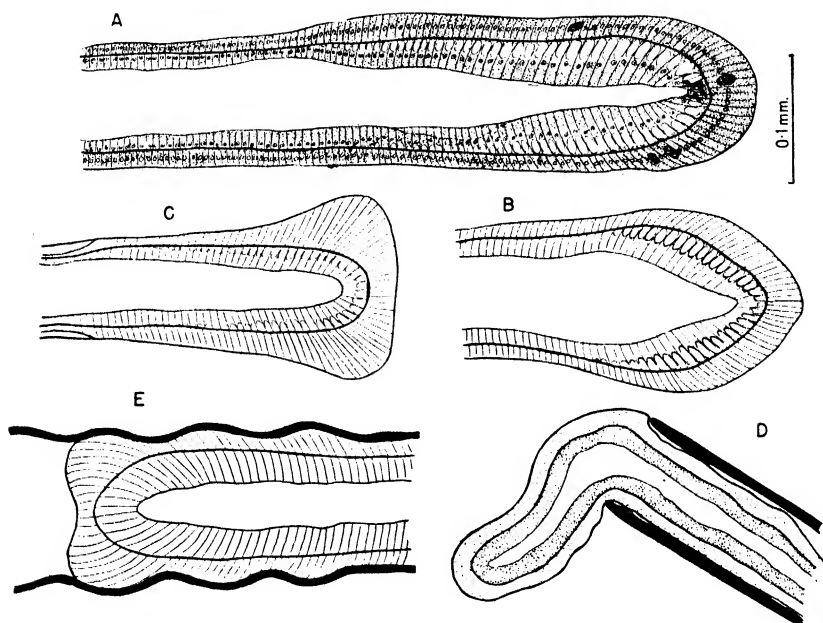
Whatever the significance of the polar vacuoles in terms of the physiology of dividing cells, they do afford a useful criterion of growth-rate. In slowly growing stolons relatively few new cells are produced in a given period, and correspondingly few cells are to be found in the vacuolated or recovery phase. In rapidly growing stolons, many more cells are formed within a given period and correspondingly more cells will be found in the vacuolated recovery phase. Each newly formed cell goes through its individual cycle in the same time. The extent and gradient in degree of vacuolation accordingly form an indicator of the biological or relative growth-rates, in contrast to actual growth-rates (Text-fig. 2A, B).

The epidermis exhibits a somewhat similar cycle. Cells in the proliferative zone are large and high columnar, and in this zone and in the recovery phase (assuming it to be comparable and coextensive with that of the endodermis) an extensive but less obvious vacuolated region exists external to the nuclei of the cells.

The flowing aspect of stolon tissue is shown even more strikingly when a piece is cut. When a stolon or branch is cut through there is an immediate closing over of the end of the endodermal layer to form a closed tube. The epidermis reacts in a similar manner but a little more slowly, as it flows up and around the tip of the endodermis. Both distal and proximal ends of a cut piece react in the same way. The response is so rapid that there is no possibility of cell proliferation being in any way involved. The condition

1–2 minutes after a cut was made is shown in Text-fig. 2E. The pseudo-fluid nature of the material is further shown by its behaviour under light glass pressure. The coenosarcal tube as a whole remains stationary and unaffected, but the tip slowly extends without significant change in shape.

When a cut is made across a stem or stolon some distance from its distal end, new growth occurs rapidly from the cut proximal end (Text-fig. 2D),



TEXT-FIG. 2. Growth of stolons. A. End of rapidly growing free stolon, showing thickened epidermis of the growing zone including a few glandular cells, and endodermis with a distal group of dense rapidly dividing cells grading into large vacuolated cells which decrease proximally in size and vacuolation. B. End of slowly growing free stolon, showing reduction in range but not extent of vacuolated zone and absence of non-vacuolated cells distally. C. End of slowly growing attached stolon, showing latero-distal spreading of epidermis. D. Stolon healing from basal end of a cut stem. E. Distal healing in cut annulated zone of a stem, showing distal endodermal fusion and flowing of epidermis (cell division not involved).

that is, the normal polarity of growth may be reversed. When such out-growths become attached to the substratum, growth may be vigorous and most of the old coenosarc and organized structures may resorb and become converted into nutrient for the new tissues.

Stolon tips readily attach to solid surfaces. Those already attached are difficult to detach without breaking them. Free stolons attach immediately upon contact, the region of attachment corresponding approximately with the extent of the recovery phase described above. In other words, the stolon surface is sticky for a limited though variable extent from the tip backwards.

When a free stolon attaches to glass, it assumes the terminal shape characteristic of creeping stolons in general. The contrast with the free stolon is shown in Text-fig. 2c. The endodermis remains unaffected, but the epidermis flows forwards and outwards as a pair of shoulders, that is, at the tip it has a tendency to spread on contact, clearly a surface tension effect.

HYDROPLASMIC STREAMING

Movement of the fluid within the hydrocoel has been generally noted. Huxley and de Beer (1923) recorded changes in contour of the coenosarc with the ebb and flow of the stream. Hammett (1943) reports the movement as streaming distally during hydranth development, proximally during hydranth resorption. Hauschka (1944) found from isolation experiments that the absence of the stream did not prevent hydranths from completing their development, and that the causal connexion suggested by Hammett did not hold.

While the present account is of *O. commissularis*, *O. geniculata* is essentially the same in this respect, and the obvious feature of the hydroplasmic streaming in *O. commissularis* is its regular reversal in direction. The rhythmic period varies with the temperature and may have from 3- to 7-minute cycle, consisting of a flow in one direction of from 1 to 3 minutes, a short period of quiescence, and a flow in the opposite direction of similar duration to the first. Cells, cell fragments, heavily pigmented cells derived from resorbed or resorbing hydranths all flow along with the stream.

The rhythmical streaming occurs in a stolon or piece of stolon in the absence of any formed or developing structures. It could be due either to a regular reversal in the direction of beat of the cilia lining the hydrocoel, or to contractions and expansions of one or both of the epidermis and endodermis. The activity of the cilia becomes most evident through the dancing movement imposed upon the travelling particles.

To determine whether cilia are in any way responsible for the current, let alone its reversal, various sections of stolons and stems were cut and studied in isolation. Only those that retained or recovered ciliary activity were employed. In no case could anything but local agitation be observed with no forward or backward current. There was no evidence that ciliary activity is responsible for the hydroplasmic streaming, and this is confirmed by observations on intact stolons in which marked ciliary activity is often seen to coincide with the absence of directional movement.

The alternative that coenosarc contractions are responsible could mean either a local activity or a general peristaltic wave. Greatly enlarged superimposed series of camera lucida drawings showed no fluctuations in stolon diameter either in the recovery zone or down the main length of the stem, but in the region immediately proximal to the recovery zone a definite expansion and contraction was evident and coincided with the reversals of the stream (Text-fig. 3B, C).

The epidermis in this region when expanded comes into fairly close contact with the chitinous cuticle, when contracted becomes separated from it by

a relatively wide space. The stream runs distally as the tube expands, proximally as it contracts. When expanded, ciliary activity and particle agitation and movement are at a maximum but when contracted no activity is discernible, due probably to sheer congestion of the solid particles. For the same reason the stream rarely penetrates the recovery zone, for cell enlargement in this region more or less occludes the lumen and leaves too narrow a channel for free movement of particles.

The rhythmical expansion and contraction of the coenosarc of this region could, however, be either passive or active, responding to pressure originating elsewhere or to the sucking up the hydroplasm by innate dilatation. That the streaming is due to and not the cause of the pulsation in the zone in question is shown by the following observations.

If stolon tips, including the pulsation zone, are cut off while the stream is flowing distally, it stops immediately in the part remaining. If it was being pushed along rather than pulled, the stream should continue to flow through the cut end, which it does not. When flowing along a stem which forks, the stream should enter both branches if propelled from below. It does not and will by-pass wide open channels to flow along a single passage, which is in conformity with a distal pull.

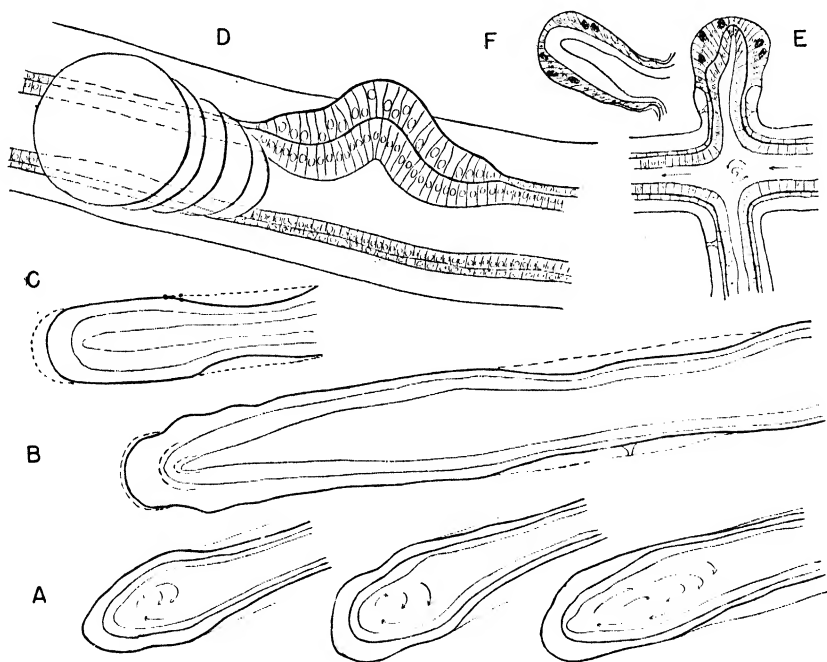
Both epidermis and endodermis are involved in the pulsation and adhere closely together. It is not obvious whether both are responsible or only one. Slight changes in thickness of the epidermis at first suggested that this layer was the contractile tissue, but other evidence indicates that it is either endodermis or the junction between the two layers that contracts and expands.

Where recovery and pulsating zones are short, and the transition gradient steep, the relative role of the two layers is more clearly seen, as in attached slowly growing lateral stolons (Text-fig. 3E, F). When expanded, the epidermis comes into contact with the chitinous perisarc. When contracted, the epidermis gives the appearance of being pulled away from its adhesion, leaving several but varying cells attached to the perisarc on each occasion. The appearance suggests that the epidermis is passively pulled between two surfaces to which it tends to adhere.

Myofibrillae have not been detected histologically, and the rate of contraction and expansion is much slower than would be expected even in the most sluggish fibrillar system. It has more in common with the contractile epithelium reported for ascidians (Berrill, 1929) and the cortical movements of teleost eggs and blastoderms (Yamamoto, 1940).

The extent of the contractile zone is variable and is much the same and in proportion to the extent of the recovery zone. In a progressively growing stolon, the contractile phase is of limited duration for any individual cell, but the faster new cells are produced the greater will be the number that will be in a given phase, whether recovery or contractile, at a particular moment. When the contractile phase is passed, the cells become quiescent endodermis, ageing slowly with time, although any stimulus that produces local cell division evokes the whole cycle anew.

There is some indication that the vacuolation-contraction cycle is less a response to a recent cell division than it is to cell growth as such, division of the cell being itself a response to the same condition. This is suggested by the appearance of the large cells initiating the outgrowth of a lateral branch



TEXT-FIG. 3. Stolon pulsation. A. Camera-lucida drawings of same stolon tip 2 minutes apart, showing changes in diameter and contour and varying extent of internal ciliary activity. B. Rapidly growing stolon, with relatively large terminal flow of epidermis; broken lines represent contour alternating with that of unbroken lines, showing slight terminal pulsation, and extensive pulsation zone proximal to thick vacuolated zone. C. Similar to B, showing two particles adhering to epidermis and exhibiting merely transverse movement with the pulsation. D. Lateral outgrowth starting from junction of stolon and base of hydranth stem; both epidermal and endodermal cells greatly enlarged as preliminary and accompaniment of proliferation. E, F. Contracted and expanded phases of a pulsation of a lateral attached stolon, showing tendency of epidermal cells to adhere to cuticle during contraction phase.

(Text-fig. 3D) and by the short period (2 hours) that elapses between the cutting of a stem and the presence of vacuolate and contractile regions.

BRANCHING

Branching occurs both from creeping stolons and from erect stems and free stolons.

Creeping stolons. Vertical branches are the first to appear, from the upper side of a stolon a short distance behind the tip. The region from which they

arise, one at a time, at *the time of origin*, is the proximal part of the contractile or pulsating zone. As the first branch grows vertically, the stolon tip has usually undergone further growth of its own, the pulsating zone following close behind the tip and leaving the vertical growing branch attached to a region that loses its pulsating property. As the stolon tip continues to grow, the distance increases between it and the base of the branch, the pulsating zone migrates forwards to a corresponding extent, and after the first branch has been left behind a certain distance, a new vertical branch forms from the upper side of the pulsating zone. This process continues more or less indefinitely (Text-fig. 1c).

The vertical branches thus formed are at first always annulated, whatever may be their ultimate fate. After several such branches have formed, a tertiary outgrowth of a stoloniac nature appears close to the junction of a vertical branch and the main stolon, the youngest junction to exhibit tertiary growth usually being the third from the tip, more rarely the second or fourth (Text-fig. 3d). In other words, it forms at a junction after that junction has existed for a certain duration of biological time.

This last type of outgrowth is always lateral and in contact with the substratum, growing out at right angles to the primary stolon. In a comparable manner, after it itself has existed for a certain period, another lateral outgrowth appears opposite it in the same plane, also in contact with the substratum. Neither group of contact stolons ever exhibits annulations.

The localization of outgrowths in the two regions just described, i.e. the pulsation zone and the branch-stolon junction, have features in common which distinguish them from other parts, namely, a wider lumen, a current system that affords greater opportunity for intracellular feeding, and probably better metabolic conditions generally as the result of more space and effective ciliary activity. If ciliary activity can be taken as a fair index of respiratory conditions, there is little doubt that such conditions are better in these two places than elsewhere, in the one due to the innate pulsations, in the other to the hydroplasmic junction pressure created by the pulsating branch terminal.

In the case of free stolons or stem terminals, the pattern of outgrowths is essentially the same as in creeping stolons. The difference here is that all branches are at first annulated, again indicating that contact with a solid substratum effectively suppresses any tendency to annulation.

In conclusion, there appears to be but one stoloniac activity in terms of cell cycle and branch formation, modified only by growth-rate and contacts.

PERISARC

Free stolons may or may not exhibit annulation. Whatever its extent, it cannot properly be discussed except in terms of the chitinous perisarc which retains the annulation even after the molding tissue has lost it.

Little study has been made of this substance and its role in the economy of hydroids. There are some unexpected properties. Weismann (1883) in-

cluded an illustration, obviously accurately presented, that Nutting (1915) doubted on *a priori* grounds, showing an outgrowth rudiment secreting its own fine perisarc and protruding through a hole in the thick chitin surrounding an old stem. Louis Agassiz (1862) in fact illustrated almost exactly the same thing for *O. commissularis*. Taking Weismann's illustration at face value, the chitinous perisarc clearly can both be laid down and dissolved under certain more or less obscure conditions.

Hammett calls attention to the elasticity of the perisarc at the distal end of nearly developed hydranths and speaks in terms, no doubt correctly, of polymerization of the secreted substance. The brittle rigidity of old perisarc in contrast to the sticky stretchiness of the newly formed material of stolons and developing hydranths suggests forcibly that polymerization does take place.

The increasing thickness of the perisarc with the age of a stem, its laminar quality, and above all, the formation of straight lamellae on the inner side of annulations, demonstrates that chitin production is continuous and not limited merely to growing regions (Text-fig. 4B).

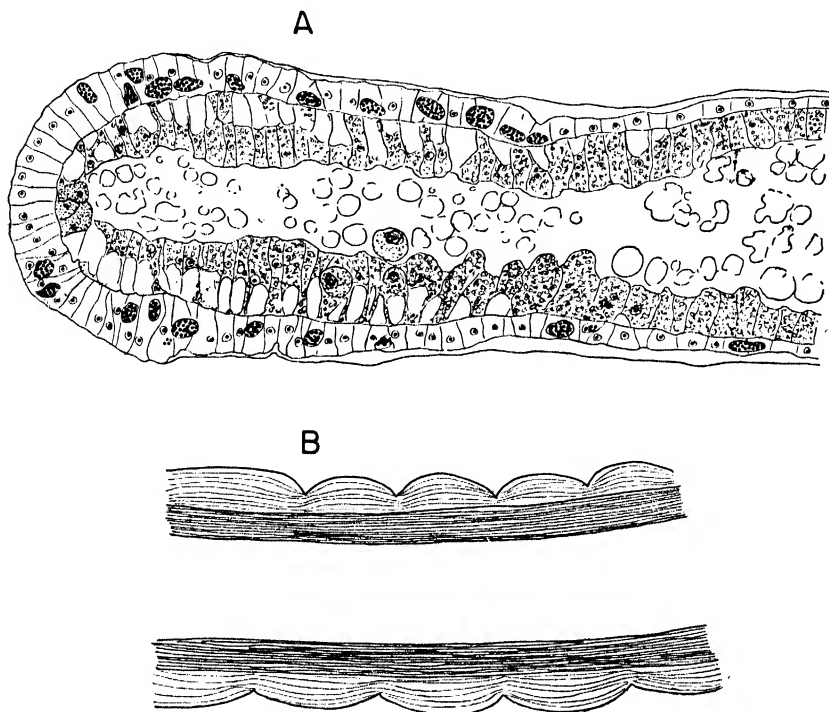
Perisarc is secreted at the growing end of a stolon, though hardly visible in the recovery zone, yet sufficiently for a very thin though non-elastic film to be present at the distal edge of the pulsation zone, gradually thickening proximally. In the pulsation zone it is no longer adhesive (unless previously attached) and is probably fully polymerized. If polymerization is rapid, as it appears to be, the various shapes permanently assumed by the chitin in conformity with the shape of the secreting tissue is explained. Straight stolons produce straight cylinders, curved surfaces produce curved perisarc.

The evidence is that chitin is secreted continuously and not only by growing regions. It is therefore either a general property of epidermal cells or else specialized cells must be present. If the former, then the property is apparently lost in the case of further specialization, as in tentacle epidermis. There is evidence, however, that specialized cells are solely responsible, and that the greater part of the epidermis is basically unspecialized.

Distinctive ovoid cells are always to be found in the epidermis of a growing stolon tip, and in somewhat smaller numbers throughout the rest of the epidermis. Similar cells occur throughout the stem epidermis, lying parallel to the surface. They are not typical epidermal cells and undoubtedly have some special function. They may well be the cells Weismann concluded were rudimentary ova, which would then need to migrate to other sites, and led him so far astray in his interpretation of hydroid reproduction. Being larger, rounder, and more granular than other cells of the epidermis, they might be interpreted as rudimentary ova if the desire to do so was excessive.

Their most characteristic feature, however, is that the cytoplasm is packed with highly refringent and insoluble granules, as clearly visible and refringent in the living cell as in preserved material (Text-fig. 4A). In the cells when alive and when preserved in formalin, though not stained, the contained granules have the same appearance and optical properties as the chitinous

cuticle, and it is difficult to avoid the conclusion that these are glandular cells responsible for the secretion of chitin. Chemical tests for chitin are crude, however, and in any case there is no guarantee that the staining properties of secreted chitin and of its mother substance would be the same. While iron haematoxylin has no special affinity for chitin as such, it does stain



TEXT-FIG. 4. A. Section of free stolon, stained with iron haematoxylin, showing numerous glandular cells with deeply staining granules in epidermis, and the three endodermal zones consisting of distal non-vacuolated cells, vacuolated zone, and proximal non-vacuolated zone. B. Chitinous perisarc of old stem, showing external loosely laid lamellae laid down during annular growth, and closely packed straight lamellae laid down subsequently.

the intracellular granules as deeply as chromatin, and darker than the inclusions of any other cells (Text-fig. 4A).

On other grounds entirely, it is plausible that these cells secrete chitin. They must have a function, they appear to be secretory, are epidermal, and are adjacent to the only epidermal secretion recognizable. Their distribution in *Obelia* and other hydroids is such as to account well for the presence and nature of perisarc structures. They also make possible a dual control mechanism for perisarc that would be difficult to account for otherwise. Special cells lay down the chitin in successive layers, while ordinary epidermal cells when dividing or undergoing rapid growth are able to dissolve it.

ANNULATIONS

The annulations of the perisarc of hydroids have long been regarded as diagnostic features of specific importance. Hyman (1940) suggests that they add strength to the stems and branches of a colony, but this is unlikely since stems more often break at an annulation than anywhere else. As formed structures they apparently have little importance to the organism, but as records of past activity of growing points they are invaluable. They are products of epidermal fluidity and chitin polymerization.

A typical annulating stolon is shown in Text-fig. 5C, D. As in simple stolons, the flow lines of the epidermis and endodermis coincide, but there is a striking difference. The epidermis, flowing distally as it grows, extends far beyond the endodermis, to such an extent that it tends to form a spherical blob or drop (Text-fig. 5E). With further growth, the tip of the endodermis enters the blob and may reduce its basal curvature (Text-fig. 5F). In the stolon shown in Text-fig. 5E the endodermal tip has passed through and widened the penultimate blob, while the epidermis has grown beyond and formed one more.

The lagging behind of the endodermis is not necessarily due to a slower rate of growth, but is at least in part the result of a slower start. The initial rudiment (Text-fig. 5A) bulges out and only after the epidermis has formed a hemispherical mass does the endodermis gather enough substance to follow (Text-fig. 5B). That is, there is an initial epidermal head start, expressed as a single annulation, unless the epidermis is in contact with the substratum, in which case the two-dimensional spreading interferes.

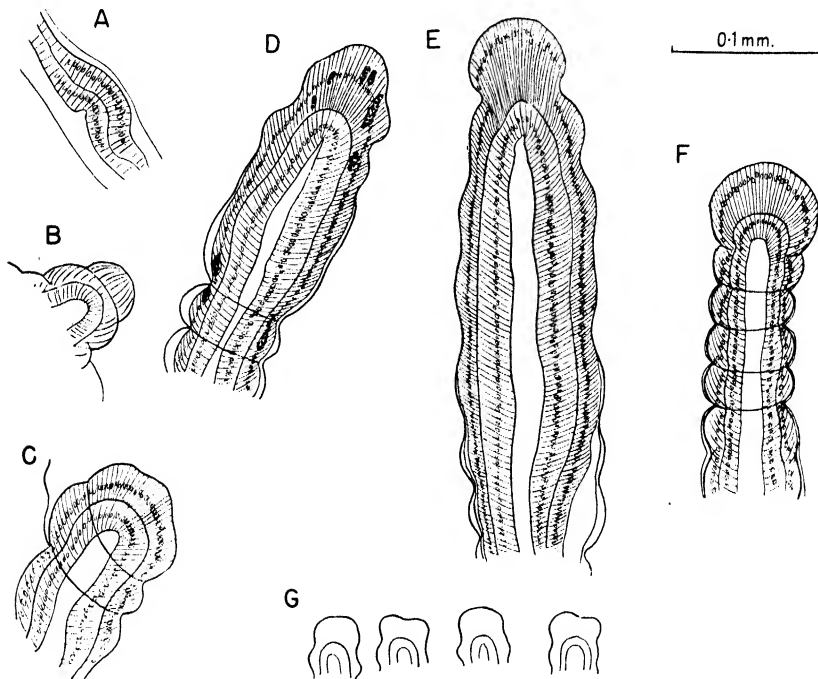
If the growth of the endodermis forces the pace a little, it catches up with the epidermis and the two tissues grow onward together as a simple stolon with a few basal annuli. Under certain conditions, such as starvation at moderate temperatures, initial growth-rates are maintained and attenuated stolons annulated throughout their length are frequently seen.

The formation of distal epidermal blobs entered from behind by the endodermal terminal presents little difficulty, but there is the additional feature to explain, that the epidermal annuli persist for a while and the perisarc annuli permanently, after the endodermal tube has passed through. To resist the flattening influence of the internal pressure and maintain superficial curvatures, a degree of surface solidification is essential.

Chitin when first secreted at the surface flows to form a barely detectable viscous film conforming to the shape of the epidermal blob. At the extreme tip where all cells are probably in process of division it is virtually non-existent. The film forms more definitely on the sloping sides, and solidification or polymerization of the film over the proximal part of the blob occurs before the endodermal tube enters it. At temperatures between 15° and 20° C. it has about one hour in which to polymerize, this being approximately the time a blob takes to form before being entered by the endodermis. If the growth-rate is relatively fast and the available time at a minimum, some

flattening is produced by the endodermal tube and annulations become shallow (Text-fig. 5E); when slow, the constrictions remain deep (Text-fig. 5F).

The succession of epidermal blobs may represent rhythmical growth surges, although it is not necessary to postulate this since a significant analogy



TEXT-FIG. 5. Annulated growth. A. Initiation of outgrowth, enlargement and division of cells of coenosarc wall. B. Early stage forming second annulus, epidermis forms blob in advance of endodermal growth. C. Two-annulus stage, with blob entered by endodermis. D. Later stage succeeding first phase of annular growth, nearing end of stolon phase, with epidermal blob again forming (glandular cells also shown). E. Similar stage with fully formed epidermal blob and lagging endodermis. F. Final blob at end of second sequence of annular growth, forming hydranth rudiment. G. Terminal contours of epidermal blob drawn 1 minute apart.

may be made between the continuous flowing of water through a small opening and its subsequent subdivision into drops by the action of surface tension forces.

With progressive growth of the coenosarc as a whole, the region in which certain annuli are formed in effect travels down the stem, while the coenosarc passes into and out of its pulsation phase and withdraws from its contact with the perisarc. The chitinous perisarc annuli in consequence enclose a straightened coenosarc. Chitin secretion continues, however, and the chitin

is subsequently deposited as straight lamellae on the inner side of an annulated outer layer (Text-fig. 4B).

The mobile fluid-like nature of the epidermal blob is illustrated in Text-fig. 5G. In contrast to the fixed contour of the base of the blob, the distal part is continually changing every few seconds from a multiple to a single curved surface and back again, the single curvature predominating.

An analogy of some significance in the light of the following sections on morphogenesis, is that of a glass blowers' terminal blob of molten glass (epidermis), with a column of air (endodermis) being blown into it through a tube.

PART II. HYDRANTH DEVELOPMENT

There is a sequence of events leading to the initiation of hydranth development which in *O. commissularis* has so far been found to be invariable.

The hydranth always arises from the end of a short stolon that has undergone the following sequential changes, namely, several initial annulations, a phase of simple (non-annulated) stolon growth, followed by a second and distal series of annulations.

The first series of annulations is due mainly to the head start of the epidermis. This overhead, however, is progressively lost as the endoderm in effect catches up, and as growth-rates and terminals coincide, straight stolon growth becomes established. After a variable period of such growth, the growth-rate of the endodermis begins to fall off again relative to that of the epidermis, and terminal epidermal blobs (and annuli) again appear.

There is a difference in the annulated growth of the first and second series. In the first it is a diminuendo, the epidermal blob becoming progressively reduced in size at the time the endoderm penetrates it, until none is formed. In the second it is a crescendo, the endodermis lagging more and more so that the terminal epidermal blob is larger each time it is formed. With the third, fourth, or fifth epidermal surge, a crisis is reached.

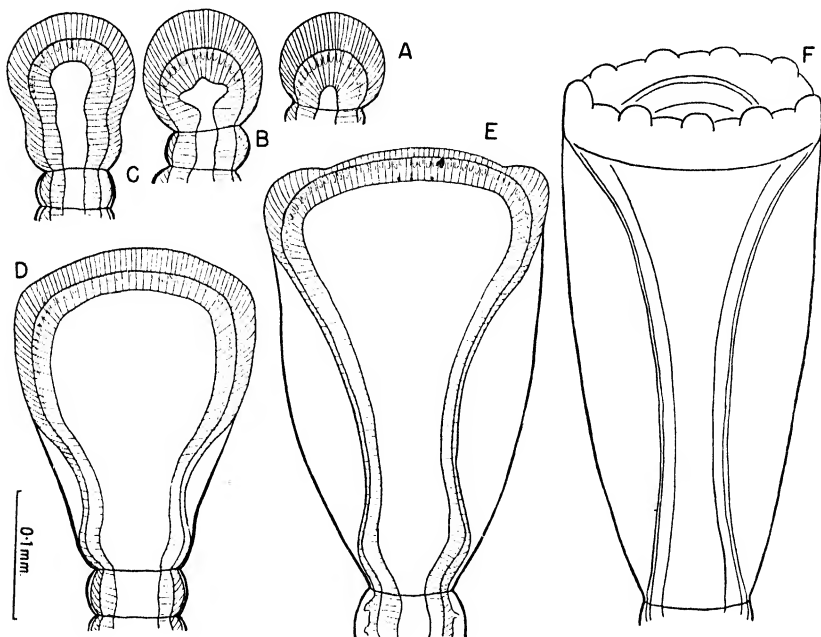
The sequence just described, culminating in the cessation of the second series of annulations, is characteristic of a relatively low temperature range, below 18°–20° C. Above this level, in most circumstances, the phase of simple stolon growth succeeding the first series of annulations continues indefinitely. The appearance is that the endodermis responds to higher temperatures a little more vigorously than the epidermis, becomes the pace-maker and enforces simple growth.

At relatively low temperatures, the absolute reduction in growth-rate of both tissues, and the relative lagging of the endodermis result finally in the formation of an epidermal blob larger than its predecessors, and entered by the endodermis even more slowly than before. Both features are important, the greater quantity of material in the unit, and the longer time in which something can happen.

The terminal unit shown in Text-fig. 5F is typical of the new starting-point, with maximum epidermal blob enveloping the tip of the endodermis.

This is the rudiment of the hydranth. The unit now grows as a whole and quantitatively exhibits a typical growth-curve or decrement until all growth ceases.

The slowing of the growth-rate of the endodermis is indicated by the disappearance of the terminal zone of relatively small rapidly dividing cells,



TEXT-FIG. 6. Hydranth development (chitinous cuticle in heavy black). A-E. Note changing range of vacuolated zone. A. Initial semi-hemispherical rudiment with terminal epidermal blob fully entered by endodermis. B, C. Enlargement and elongation as cells become added from the anterior disk to the proximal cylinder. D, E. Progressive growth, limitation of vacuolated cells to anterior disk, proximal formation of pulsation zone causing detachment of coenosarc from the perisarc. F. Slightly later stage at same scale showing complete separation of coenosarc and perisarc, and conversion of marginal rim of disk into rudiments of primary and secondary tentacles.

and the substitution of dividing cells of maximum size and vacuolation. The effect is to give expansion a lateral as well as a distal direction, resulting in the slow formation of a flask-shaped structure (Text-fig. 6A, B, C).

At the same time, apart from change in diameter, which increases at the distal end, and in the absence of a centre of maximum growth-rate, the growth sequence of cells is basically similar to that in stolon growth. Cell division continues in the area extending from the distal centre to the shoulder slope, vacuolation of the endodermis being at a maximum in this region, the epidermis being comparable in its own way. Behind the shoulder, tapering

toward the neck, vacuolation decreases and disappears near the junction with the annulated stalk (Text-fig. 6c).

As growth proceeds, the distal disk of cells becomes wider. More cells pass from the edge of the disk into the distal part of the slope. Vacuolation disappears in cells farther and farther from the junction with the stem. Epidermal cells become progressively less columnar in company with those of the endodermis.

With the proximal disappearance of vacuolation, a zone appears that corresponds in relative location to the pulsation zone of the stolon, and with its appearance the pulsations commence. At first the extent and the amplitude are such as to be barely detectable. With progressive distal addition to the area, both the extent and the amplitude increase, the rhythmical movements become obvious, and the epidermis is pulled away from the chitinous perisarc (Text-fig. 6D, E).

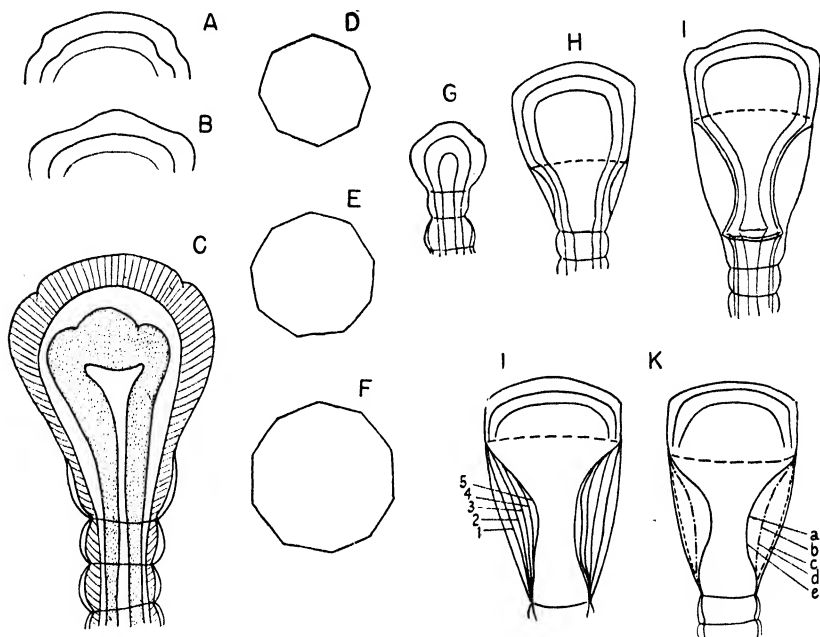
The pulsations in the hydranth have been reported already for later stages in *O. geniculata* by Hammett (1943), who regards them as the result of hydroplasmic ebb and flow originating elsewhere in the colony. To test this in *O. commissularis*, various stages of developing hydranths were isolated by cutting through the stem in the distal annulated zone. The reversing hydroplasmic stream in the intact stems ceased immediately, while the rhythmical contractions and expansions in the hydranth buds continued indefinitely. Accordingly there is no reason to doubt the existence of active pulsation in the hydranth itself, while the manner of formation of the zone and the identity of rhythm rates leave little doubt that the phenomenon in hydranth and stolon tip is the same.

The pulsation zone is gradually extended distally until the bud consists of a long pulsating cylinder of flat and cuboidal cells, surmounted by a cap of columnar epidermal and endodermal cells that forms a right angle junction with the cylinder. The cylinder becomes the stomach of the hydranth, and the pulsations persist almost until the hydranth as a whole becomes a functional entity. The extent of growth of the whole from the bud rudiment to the present stage is shown to scale in Text-fig. 6A-F. Text-fig. 7J shows five consecutive positions of the cylinder wall in process of contraction, Text-fig. 7K five positions occupied one minute apart.

As the round bottom of the inverted flask (Text-fig. 8A) becomes converted into a flat bottom, a new crisis is reached. No further cells are added to the slopes to augment the pulsation zone, while other events take place at the marginal junction. The profile of this stage is shown in Text-fig. 8B. The cylinder is surmounted by an extremely straight-sided ridge or flange, which in turn surrounds a slightly convex disk. Both ridge and disk, in contrast to the pulsation zone, consist of high columnar cells in progress of slow division. The inner surface of the endodermis of this region bears very active cilia.

The interest in this stage concerns the marginal rim. Seen in lateral profile, nothing appears remarkable, but examined from the more difficult angle of

above or below, the circumference is seen to be no longer circular but many-sided. The number of sides varies from 8 to 12, according to the size of the hydranth bud at this stage. Imperceptibly, the circular outline of a slightly earlier stage flattens equidistantly to form 8, 9, 10, 11, or 12 sides (Text-fig. 7D-E), the length of a side being approximately the same in all, and the



TEXT-FIG. 7. Hydranth development. A, B. Alternating contours of distal end shortly before tentacle initiation. C. Similar stage with endodermis shrunk as consequence of narcotization, showing marginal growth of epidermis and endodermis independently. D, E, F. Distal end-views of stage I and Text-fig. 6E, showing polygonal form, and variation in number of sides with length of circumference. G, H, I. Three stages of early development drawn to same scale as in Text-figs. 8 and 9. J. Pre-tentacle stage showing five consecutive contours of cylinder wall during contraction. K. Same stage, showing position of cylinder wall during expansion and contraction $1\frac{1}{2}$ minutes apart.

number of sides being directly proportional to the length of the circumference. This foreshadows tentacle formation, and the number of tentacles that will be formed is twice the number of sides, whatever that may be.

At 20° C. it takes about 4 hours to pass from the stage where polygonal structure is suspected to the definite configuration, a relatively long period in view of the rapidity of subsequent changes.

As the cylinder alternately expands and contracts, the contour of the distal region alternates between those shown in Text-fig. 7A and B. At one time the marginal thickening appears to be epidermal, at the other it seems to be endodermal. It is difficult to determine which of the two layers is really

responsible for the variable thickness of the whole, even when vitally stained with brilliant cresyl blue or with neutral red. However, a prepared slide was discovered representing stained specimens that had clearly been narcotized preparatory to fixation, and while formed structures and the epidermis throughout appeared to be in excellent condition, the endodermis of rapidly growing regions was shrunken or withdrawn. In one instance, at the stage in question, a space had appeared between the two layers (Text-fig. 7 c), and it is evident that in both epidermis and endodermis growth has resulted in the differentiation of a thick marginal rim and a central dome. Both layers are involved.

After the distal polygon has been definitely established, a relatively, even remarkably, rapid transformation occurs. Each epidermal corner becomes rounded, again like a bead induced by surface-tension. Only a few minutes later the unorganized territory remaining between adjacent corners also draws up into small hemispherical masses (Text-fig. 6f, 8c).

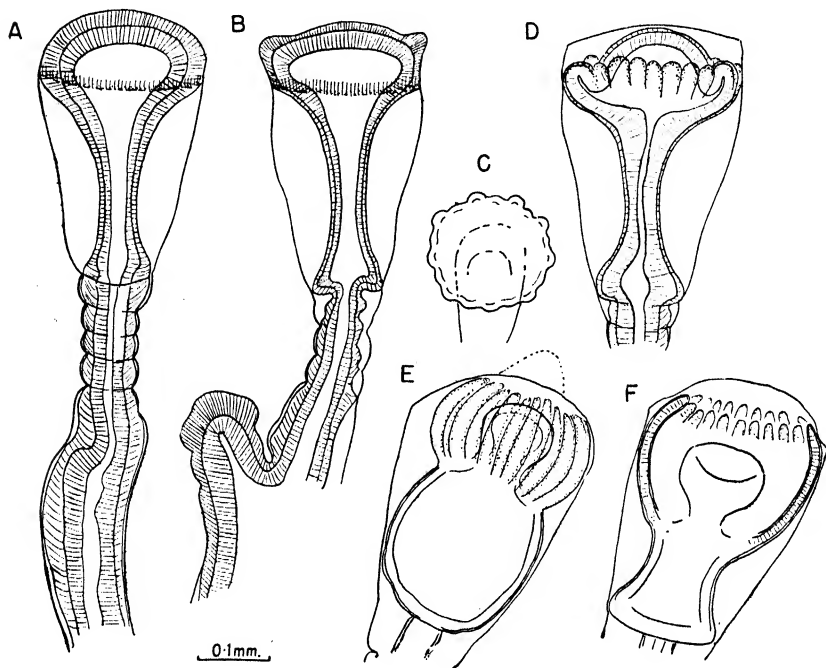
The row of beads thus formed at the margin of the disk represents the full number of tentacles that are to be formed, those arising from the corners giving rise to the more or less horizontal tentacles of the expanded hydranth, the intermediate ones becoming more or less erect.

The tentacles of *Obelia* are small and as they are initiated on a correspondingly small scale, they are less suitable than those of many other hydroids for an analysis of tentacle histogenesis. At the same time, a tentative analysis is as follows. Local proliferation of marginal endoderm results in the delamination of masses of very small cells, a similar process occurring in the adjacent epidermis. The small endodermal cells enlarge individually, and constitute the endodermal chordal cells of the tentacles without further increase in number. As the epidermal covering becomes thrust out, the small cells of epidermal origin are also drawn out, giving rise to the nematocysts.

The final phase of hydranth development occupies but a few hours, and consists mainly of differentiation processes in the three zones already established (Text-fig. 8d). The central dome grows into a thin-walled manubrium, opening distally to form the mouth. The marginal beads rapidly grow out as the tentacles, while the stomach forming from the wall of the cylinder acquires typically pigmented gastrodermal cells, with inclusions that stain intensely with neutral red.

Simultaneously, movements commence indicative of the functional differentiation of muscle-fibres. Twitch contractions appear in the tentacles, occupying merely a fraction of a second. In the stomach, movements occur which change the shape rather frequently, each transformation occupying but a few seconds (Text-fig. 8e, f). Similar movements develop in the manubrium though somewhat later. While these enteric contractions are slow compared with a tentacle twitch, they are very fast compared with the rhythmical pulsations described earlier, and there is no possibility of confusion between them.

Hammett (1943) has recorded for *O. geniculata* the necessity of rhythmic surging of the whole hydranth for the final stretching and rupture of the thin sticky perisarc covering its distal end. In *O. commissularis* it is not certain that a coat of quite such consistency is present. In many cases the hydrotheca retains its polygonal character, though it is slight and often disappears.



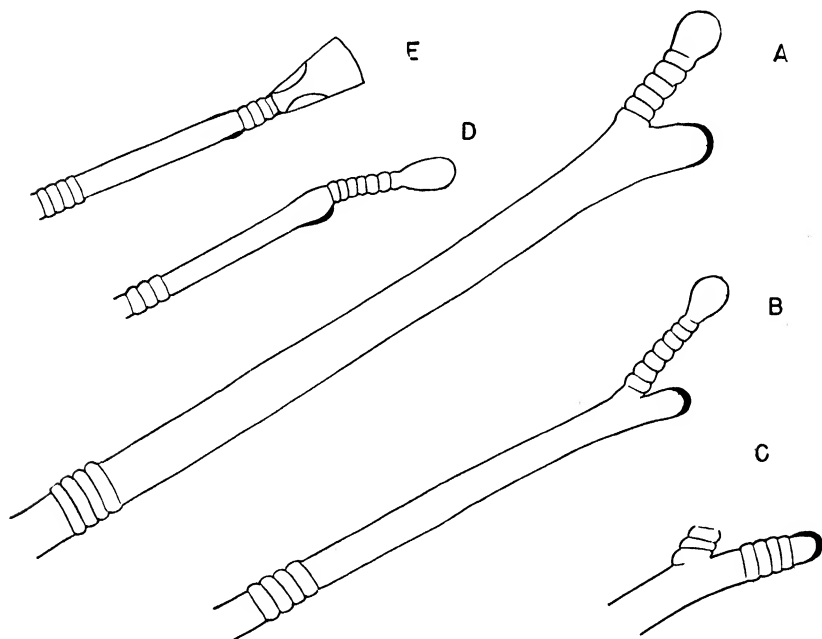
TEXT-FIG. 8. Later development of Hydranth. A, B. Late pre-tentacle stages, showing origin of new terminal growth from wall of distal part of non-annulated or internodal stem. C. Distal view of initiation of primary and secondary tentacles. D. Lateral view of stage immediately following that of Text-fig. 6F. E, F. Later stages with dilated and contracted body-wall, showing elongating tentacles and formation of manubrium.

With the opening of the mouth, the large pigmented gastric cells derived from the resorption of hydranths elsewhere in the colony and accumulated in the cul-de-sac of the developing hydranth, escape to the exterior. In young hydranths they may be seen as a ball of yellow-orange material, opaque by transmitted light, held between the tentacles.

It was mentioned earlier that hydranths usually do not start to form unless the temperature is below 20° C. If after development has started the temperature rises even several degrees above this threshold, development continues to completion. At temperatures in the region of 25° C., however, the hydranth reaches the functional state and then usually resorbs immediately, indicating a greater susceptibility of fully differentiated specialized cells to high temperatures than either cells in the developing system or in the unorganized

coenosarc. The process of hydranth resorption has been adequately described by Huxley and De Beer (1923).

One phenomenon remains, of some interest in *O. commissularis* itself and of considerable interest when comparisons are made with other species and genera. This is the manner of terminal growth of main and subordinate



TEXT-FIG. 9. Terminal branches of colony, all to same scale. Heavy crescent represents distal growing zone. A. Terminal of a main stem, B, of secondary branch, showing similarity of growth form and difference in stem diameters. C. Continued growth of distal end showing primary annulation phase and commencement of internodal simple growth. D. End of branch of 3rd and 4th order, showing belated initiation of terminal growth. E. Remote lateral branch terminal with developed hydranth and suggestion only of new growth zone.

branches. The main stems of a colony and the stems of the next order are similar except in diameter (Text-fig. 9A, B). In these two cases, when the second annulation series is complete and the hydranth bud has reached the flask-shaped stage, new stolonial growth has emerged from one side of the anterior internodal stolonial region. This in turn undergoes its first annulation series and then converts to stolonial growth (Text-fig. 9C).

In terminals of branches of the third or fourth order, a difference appears. When the hydranth bud has reached a comparable stage (Text-fig. 9D) the new terminal growth arising from the distal non-annulated region has barely started, while in branches even more remote from the main stems hydranth development may be complete before the new growth starts (Text-fig. 9E),

and in fact may arrive at a condition where there is a functional terminal hydranth and no further growth at all.

PART III. GONANGIUM AND MEDUSAE

The production of gonangia raises the following questions. Where and when do they arise, and why? What are the formative processes initiating their appearance? How does the early developmental phase differ from that of a hydranth, what are the developmental consequences, and what other factors intervene to bring about further differences?

PLACE OF ORIGIN

In this species the gonangia arise only in the basal and central part of a colony, that is, from the older parts. They arise generally from established junctions, regions where a stem junction already exists. From such places gonangia are second outgrowths, although second outgrowths need not be gonangia. In the same general region of a colony, though always toward the proximal end of a branch, gonangia may arise from the unbranched internodal pedicel of a hydranth.

Since junction zones tend to become larger with age, the diameter of such a zone might relate to the size of outgrowths emerging from it, and this in turn be a determining factor. On the other hand, those arising from simple hydranth pedicels would remain unaccounted for, and in any case the initial gonangial protrusions do not appear to differ from those leading to hydranth development. It is therefore more likely that the primary determinant is metabolic rather than physical or dimensional. If the lower growth-rate of lateral branch terminals is taken as a criterion of metabolic conditions, it indicates a lower growth tendency in the basal parts compared with the apical parts of a colony. The rates of stolonial outgrowth from pieces of stem isolated from various regions confirm this.

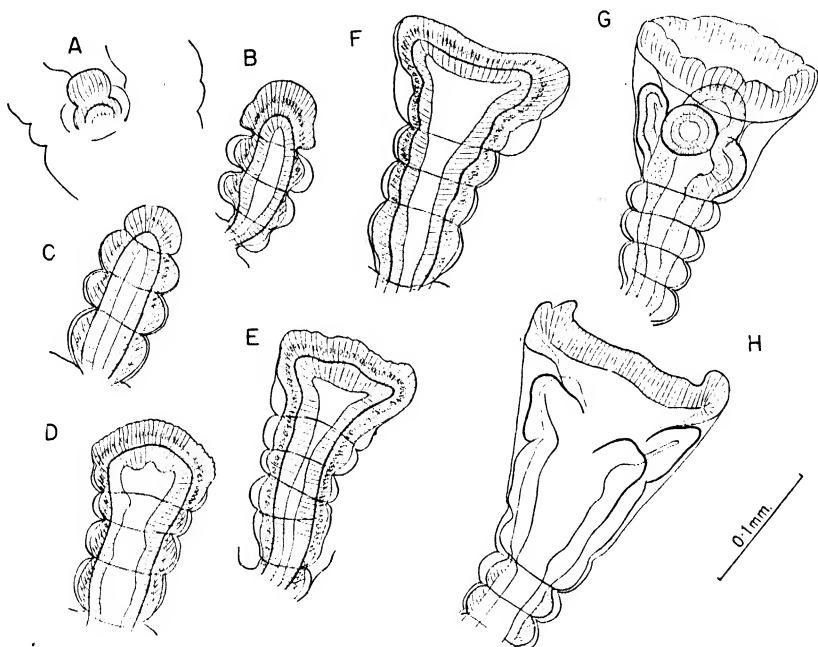
GROWTH SEQUENCE

While the initial protrusions appear to be similar for hydranth and gonangium, the succeeding growth sequences are very different. The hydranth sequence consisted of a series of annulations of diminishing diameter, changing to simple stolonial growth culminating in annulations increasing to form the hydranth rudiment.

In the gonangial sequence, annulating growth commences as before, but the endodermis not only fails to overtake the epidermal blob, but lags so that the terminal cap enlarges with each annulation. Three or four annulations occur in this manner, corresponding in kind to the second series of the hydranth stalk, but forming here as the primary series (Text-fig. 10A-E). Sometimes the number of annulations may be much greater, but it is still a crescendo and the final state is the same.

GONANGIAL RUDIMENT

As the final surge occurs, which produces the gonangial rudiment, another difference appears. The final unit is flatter and wider than the hydranth rudiment, and from the first consists of a considerably larger number of cells (cf. Text-fig. 6A and 10D). This difference was noted by Weismann (1883)



TEXT-FIG. 10. Development of Gonangium. A. Initial stage. B, C, D. Primary annulation growth phase with expanding diameter culminating in gonangial rudiment. E, F. Growth of rudiment, especially of disk. G, H. Initiation of medusa buds from wall of anterior cylinder, and consequent shrinkage of distal endodermis (stippled in G).

and by Hammett (1943), who compares the two primary caps with a cylinder (hydranth) and tam-o'-shanter (gonangium). However, this is a difference that develops rather than one which at first exists.

If we compare the formative blobs at the time of endodermal entrance, the difference is less striking. It is none the less significant. The distal flow of the epidermis is reduced and there is greater lateral spreading, so that more cells lie in the transverse plane, and the greater total number of cells present in the initial gonangial rudiment both lead to the development of a larger final structure, final size being a reflection of initial scale of organization.

DEVELOPMENT OF THE GONANGIUM

The unit which continues to enlarge as the developing gonangium shortly consists of a convex cap and two semi-annulations which remain too shallow to prohibit the contained coenosarc from participating in the formative zone (Text-fig. 10F).

Both hydranth and gonangium rudiments follow a growth-rate curve to a final state of equilibrium or cessation of growth. On the reasonable assumption that the growth-curves are typical, then stage for stage the gonangium is larger than the hydranth, or at similar sizes is developmentally younger. This is borne out by a comparison of the final length and mass of hydranth and gonangium. It is also significant that at the same temperature the relatively large gonangium, as well as can be determined considering the vagueness of the developmental end-point, develops in approximately the same time as the hydranth.

In essence, therefore, the gonangium in its early development may be regarded as a developing large hydranth, commencing to form a wide distal disk, and proximally a wide tapering enteron. If this interpretation is valid, why does not a gonangium exhibit more of hydranth structure, and why does not a hydranth produce medusae?

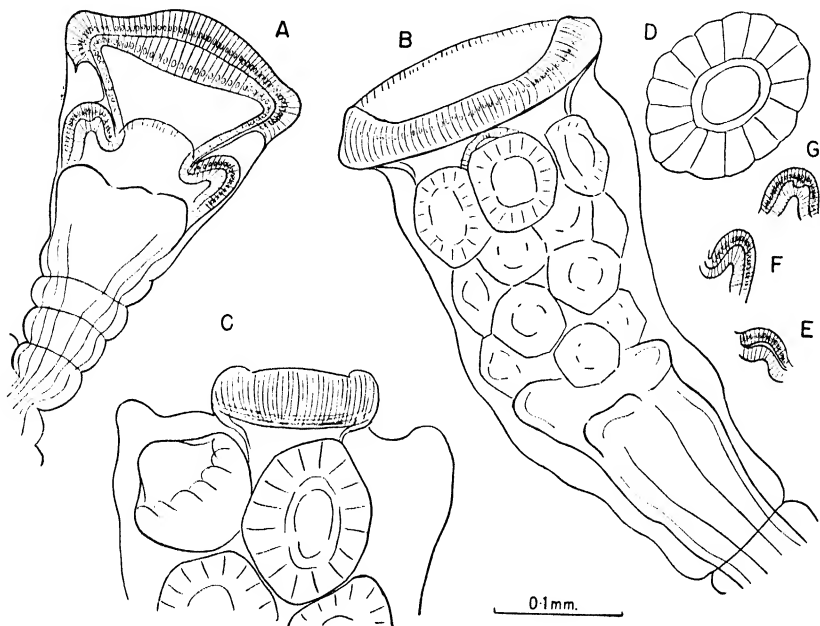
The answer to the second question is that during the critical development phase, the hydranth is not large enough for the initiation of medusa disks, and a second opportunity never arises since no growth occurs in the functional hydranth.

On the other hand, a developing gonangium forms medusa rudiments primarily because there is sufficient territory in its wall. This matter is discussed further in connexion with medusa formation and development. In the present connexion, what is important is the effect of medusa initiation upon the continuing development of the gonangium as a potential hydranth. Medusa initiation is precocious, that is, it occurs during the developmental period of the parent individual, and not later as in some other hydroids. The consequences are to be seen in the later development of the gonangium.

The epidermal terminal disk grows and forms a marginal ridge surrounding a central plateau (Text-fig. 10H), equivalent to the hydranth stage preceding tentacle demarkation. Without further change, however, it continues to grow, finally shrinking to a very considerable degree (Text-fig. 11A, B).

Up to the time of the initiation of the first group of medusae, the endodermis is in close contact with the terminal epidermis. If it remained in contact, further mutual growth might be expected to result in formation of tentacles and manubrium. As it happens, the terminal endodermis becomes drawn more and more into the formation and nourishment of the anterior medusae. The actual stage at which distal endodermal shrinkage becomes obvious is somewhat variable. In the example shown in Text-fig. 10G it is already very marked, and the gonangial cap is virtually epidermis alone. In the one shown in Text-fig. 11A, somewhat larger; the process has not yet

commenced. Sooner or later, unable to serve two masters, the endodermis progressively abandons its contact with the epidermal plate, and the latter is clearly unable alone to develop tentacular or manubrial structure. Frequently the epidermis shrinks as rapidly as the endodermis, and the two layers remain in contact.



TEXT-FIG. 11. Development of Gonangium and medusae. A. Later stage with distal endodermis still in contact with epidermis, formation of distal ring of medusa buds and initiation of second ring proximal to it and progressive widening of stem coenosarc so that it becomes included in the developing gonangial unit. B. Late stage in gonangial development with distal epidermal disk at its maximum state, with successive graded rings of medusa buds. C. Distal part of gonangium at maximum size, showing pre-liberation medusae, and shrunken epidermal disk. D. Medusa at maximum size within gonangium, awaiting merely functional expansion. E. Initiation of medusa bud. F. Medusa bud rudiment. G. Medusa bud with epidermis thickening distally to form entocodon.

The gonangium is consequently to be regarded as a hydranth large enough to produce medusae but with its own later development aborted by the competitive demands of the medusae themselves.

MEDUSAE

The formation of medusae and the later development of the gonangium are too inextricably associated for separate treatment to be profitable.

The wall of the gonangium tapers in diameter from the wide distal disk to the narrow stem at the base. The first medusa buds to appear are four

that develop in the same annular zone just below the disk where the wall is widest (Text-fig. 10G, H). By the time these are well established, the gonangium has undergone further growth, both in diameter at all levels and in length. In fact both the annulations of the stem and the contained coenosarc widen during the process, and the formative tissue of the gonangium clearly includes the coenosarc virtually as far as its junction with the main stem (Text-fig. 11A). As the zone immediately below the first four medusa buds attains a diameter or girth similar to the more anterior zone at the time of medusa-bud initiation, a second ring of buds is initiated (Text-fig. 11A). The process continues, without significant increase in distal diameter, but with a progressive lengthening of the whole unit and a progressive increase in diameter farther and farther down the cylinder and stem, with whorl after whorl of medusa buds becoming initiated as enlargement of the wall makes it possible (Text-fig. 11B).

The epidermal disk maintains its maximum size for a while (Text-fig. 11B), but finally it begins to shrink until once more it comes to lie around the endodermal component (Text-fig. 11C). This is to be expected, for nutrition of the epidermis is maintained by the endodermis, and the earlier withdrawal of the endodermis left the epidermal cap in a state of chronic starvation.

Medusa buds of *Obelia* are comparatively small and are far from being the best material for the study of medusa morphogenesis. The essentials, however, can be determined and are in conformity with the earlier observations of Weismann (1883) and Agassiz (1862). The three earliest stages are shown in Text-fig. 11E, F, and G. A shield-like disk of tissue consisting equally of epidermis and endodermis, seen in profile in Text-fig. 11E, grows out from the parent wall and grows anteriorly as a deepening fold (Text-fig. 11F). It is notable that the rudiment develops from a relatively broad, flat area of wall, in contrast to the terminal growth of stolon, hydranth, and gonangium. There is no distinction into a distal cap and proximal cylinder. Also, the component cells of the whole are comparatively small and numerous for the size of the rudiment, indicating a relatively rapid rate of division. Finally, it is significant that the rudiments arise from wall tissue which itself is growing to a marked degree.

When the medusa bud has acquired a two-layered hemispherical form, the central distal region of the epidermis thickens. The rate of growth at the centre is or becomes greater than elsewhere and to this extent at least the development of the medusa bud resembles that of the gonangium, hydranth, and stolon. In this case, however, the epidermal thickening results in a proximal and not in a distal epidermal extension. The thickened region becomes two-layered and the inner layer is consequently segregated as the entocodon. This structure is the key to medusa development, as distinct from its initiation, and has been recently discussed at length elsewhere (Berrill, 1949). If no entocodon can be segregated, no medusa can be formed. Only the entocodal mass exhibits the tetra-radial pattern uniquely characteristic of

medusa organization. The details of its later development are not followed here, except to indicate the final size of the medusa attained before the functional expansion which occurs at the time of liberation. This is shown in Text-fig. 11D at the same scale as the rudiment. All medusae of this species are liberated with sixteen tentacles.

Within the limits of observational accuracy, it is notable that at a given temperature the time taken for a medusa to develop from initiation to liberation is approximately the same as the developmental periods of the hydranth, and of the gonangium up to its stage of maximal development as such (at 18–20° C. it is about 24 hours). It is also significant that in each of the three cases the final diameter at the end of its developmental period is approximately four times the diameter of the earliest definitive rudiment. If these two statements are accurate, and they appear to be reasonably so, we get the implications that the initial scale of organization determines the final size at the end of development, that the material in each follows a curve of growth representing a definite multiplicative value of its mass, and that each unit of mass grows to the same extent in the same time, and therefore that growth-rate and developmental time are independent of the initial total mass.

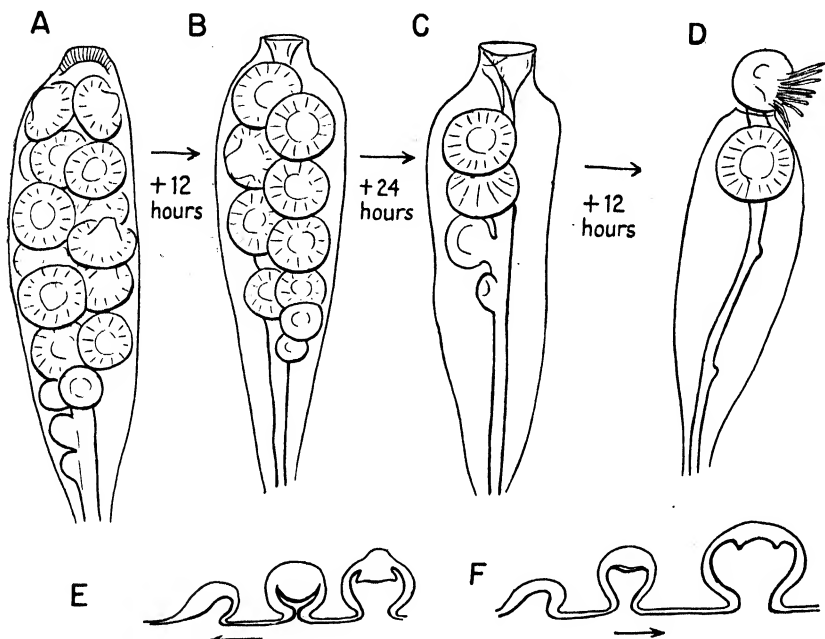
During the development of the medusae while attached to the blastostyle of the gonangium, regular contractions and expansions may be seen similar to the pulsation phases of stolon and hydranth. The time phases are similar, and there is the same alternating occlusion and dilatation of the hydrocoel (Text-fig. 12E, F). While this is probably due to a basic biological property of cells in a certain phase connected with growth, there is no doubt that in this case above all a useful function is performed, serving to draw nourishment from the hydroplasm streaming through the blastostyle, and effecting a regular metabolic exchange.

The hydroplasm streams in and out of the blastostyle hydrocoel in the same manner as it does in the developing hydranth. The contractions and expansions appear to be effected by the endodermis near the distal end. The result is that the developing medusae are maintained effectively and the stream is active usually until the last medusa has been liberated, although the innate pulsations of the developing medusae themselves probably are an adequate means of creating a communal feeding current.

When colonies bearing large numbers of large gonangia are kept under starvation conditions, so that any continued development must necessarily be at the expense of other tissues, the same phenomenon of differential resorption appears as that described by Huxley and de Beer for other hydroid species (1923) and for the ascidian *Perophora* (Huxley, 1921). Fully formed hydranths regress, developing hydranths complete development and then regress, while the medusa buds go on developing and finally all become liberated. Developing tissues are clearly in a very different physiological state from the same tissues fully developed. In this case the result is clearly both an inevitable one of colonial economics, and one of obvious value for the survival of the species. In fact, the medusa buds in completing

their development may bring about the resorption of most of the stem coenosarc.

Liberation of medusae is effected not so much by the breaking of the medusa stalks down the length of the blastostyle, but to the continued growth in length of the blastostyle itself in a distal direction. As it continues to



TEXT-FIG. 12. A-D. Liberation of medusae. A. Fully developed gonangium with cap at minimal state, with all medusae developed or initiated. B, c. Same gonangium 12 and 36 hours later respectively showing progressive elongation of stem or blastostyle distally, and successive liberation of medusae. D. Twelve hours later, with one medusa in process of detachment, and last medusa still within gonangium, with two abortive medusa rudiments on elongating residual blastostyle. E, F. Three stages of developing medusa buds showing contracted and expanded conditions; arrows show direction of hydroplasmic stream of blastostyle.

grow, the distal medusae are more or less pushed through the opening left at the gonangial tip by the shrinkage of the epidermal cap. As these break free, further extension carries the next group out, and so on until the last medusa is borne up to the threshold. The progression is shown in Text-fig. 12A-D.

The liberated medusa immediately expands. There is no sign of gonads at this stage. It is equipped with 16 tentacles and 8 lithocysts. At 20° C. there are from 6 to 10 swimming contractions in about 3 seconds, followed by a rest pause of similar duration. The medusa rotates clockwise, completing a full circle with every 7 or 8 contractions.

SUMMARY

The growth cycle of *O. commissularis* McCrady is analysed in terms of tissue mass and shape, cell number and cell transformations.

A detailed analysis is made of stolon growth typical of both free and attached stolons. Cells generally pass in succession through a phase of rapid non-vacuolate division, slower vacuolate division, a vacuolate recovery phase, a pulsation phase, and a resting phase. Cells in the resting phase may recommence the cycle at any time.

Chitin is almost certainly secreted by specialized glandular cells laden with highly refringent granules. Chitin is at first secreted as a viscous film, but rapid polymerization makes initial curvatures permanent features, such as annular rings. Local growth of epidermis in some way dissolves thick polymerized chitin.

All branches growing from a free or attached stolon and not forming at pre-existing junctions, undergo a phase of primary annular growth, a simple stolon growth phase, and a second annular phase. Annulations are formed as the result of the epidermis rhythmically forming a series of terminal blobs in advance of the growing tip of the endodermis which penetrates them successively but belatedly. In the first series the blobs decrease in size until both tissue layers grow evenly as stolon growth, the second series increase in size until a critical threshold size is attained which represents the hydranth rudiment.

The hydranth rudiment grows as a unit. Cells for the most part divide at the distal end to form a disk of increasing diameter. Cells are progressively added from the disk margin to the wall of the cylinder or body, where they pass through the pulsation phase and pull away from the surrounding perisarc. There is no further growth of the wall itself. The anterior disk becomes polygonal in outline, and the number of sides is always equal to half the number of tentacles to be formed, the tentacle number varying from 16 to 24.

Gonangia develop only from stem junctions already formed. Growth is annulated as in the case of the hydranth, but the epidermal blobs increase in size from the first and the final blob which represents the gonangium rudiment proper is somewhat larger than the comparable hydranth rudiment and is also relatively wider in the transverse plane.

The gonangium develops in the same manner as the hydranth up to the stage at which a hydranth would be about to form tentacle rudiments. At this time in gonangial development, the formation of medusa buds from the anterior body-wall immediately below the tentacle disk is so demanding that either one or both tissue layers of the anterior disk begin to shrink. The gonangium is interpreted as a relatively large hydranth whose later development is aborted by the precocious onset of medusa-bud formation.

Medusa buds arise from the gonangial wall while it itself is in progress of growth and arise in series as annular groups commencing anteriorly as the gonangial wall progressively attains an adequate girth.

The medusa bud arises directly from the wall and not from the terminal of a growing stolon as in hydranth and gonangium. The cells are small, indicating relative rapidity of division, and an entocodon, essential for medusoid organization, is formed by apical thickening of the medusa-bud epidermis, followed by segregation.

As medusae develop, the blastostyle to which they are attached grows and elongates towards the anterior end, thereby carrying medusae in effect through the distal aperture of the ripe gonangium, in order of their seniority. Upon liberation the medusae all have sixteen tentacles and no sign of gonads.

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On the Function of the Interstitium of the Testis

The Sexual Cycle of a Wild Bird, *Fulmaris glacialis* (L.)

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With one Plate

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INTRODUCTION

BENOIT (1927, 1929) described two phases of the interstitial or Leydig cell of the testis of cockerels. He stated that in the embryo chick these cells become lipoidal in character. Later, in the young bird, they are 'rich in fat-bodies' and relatively poor in mitochondrial elements. Then, at the approach of spermatogenesis, a cellular transformation occurs until the Leydig cells have lost most of their lipoids and contain an abundant cytoplasm extremely rich in fuchsinophil substances. Benoit (1924) showed that the endocrine function of the testis remains unimpaired after the destruction of the tubule elements by Röntgen radiation, and presented quantitative evidence (Benoit, 1922) that the volume of interstitial cells runs hand in hand with the development of the secondary sexual characters.

Sluiter and van Oordt (1947) injected cockerels with gonadotrophic hormone and, with controls, demonstrated Benoit's two adult Leydig cell phases (lipoid and fuchsinophil). They did not observe Benoit's reported transition. They redescribe his two main types and report a third as well. These are:

1. The lipid cell, which Sluiter and van Oordt believe not to be directly concerned with hormone production.
2. The non-lipoid cell, exhibiting numerous granular and filamentous mitochondria, which they term 'secretory cell A'.
3. Another non-lipoidal cell which contains large numbers of mitochondria and, in addition, one large vacuole which sometimes contains a crystal-line substance. This is termed 'secretory cell B', and it evolves, they

say, from type 'A': After injections of gonadotrophin the number of the non-lipoid cells rose sharply, and head appendages increased greatly in size. Sluiter and van Oordt believe that these 'secretory' cells produce the male sex hormone.

In the course of other studies of sexual periodicity the present author has been able to recognize the two chief cell-types of Benoit and Sluiter and van Oordt, but, working with wild species in which the sequence of sexual behaviour is known, he has had to disagree with Sluiter and van Oordt's interpretation of the function of the cell-types. The present contribution suggests that if the endocrine function is located in the interstitium, it is the lipoidal cells, and not the so-called 'secretory' non-lipoidal ones, which influence the bird in its prenuptial sexual behaviour. The species, *Fulmaris glacialis* (L.), used in this investigation does not develop measurable secondary sexual characters such as head-appendages. Its interstitial cytology, therefore, will be equated with its movements, its taking up of territory, and its display, copulation, ovulation, and moult. At present it is not denied that Sluiter and van Oordt's 'secretory' cells may possess some endocrine function, but it will be stressed that they appear in numbers only at and after the peak of spermatogenesis. Therefore they will be here termed *fuchsinophil* cells as distinct from the *lipoid* cells and *juvenile* cells (which will be described).

MATERIAL AND METHODS

Fulmaris is a petrel which ranges widely throughout the Arctic, North Atlantic, and North Pacific, and breeds plentifully in suitable areas in North Britain. It was chosen for the present study because it can be obtained with comparative ease during the periods of profound gonad modification, because it has a sharply defined single annual breeding season and fairly definite annual movements, and because its breeding habits and movements are well known and easy to check. It is not a true migrant, but a 'dispersive' species. In North Britain its behaviour may be broadly summarized as follows: in the last fortnight of August to early September the breeding population and young leave the nesting cliffs after the breeding season. They disperse over the sea. The first home-coming birds appear back on the breeding cliffs in November and December, dates of arrival varying from population to population. At first the numbers fluctuate, but by mid-February great numbers appear to be stationary in traditional breeding areas. Some breeding sites seem to be held by the same paired birds from the time of arrival (R. Richter, personal communication). From the time of their arrival the birds undergo a noisy display which probably has sexual significance. Apparent copulation has been observed as early as 6 April in a population where eggs were first laid about 17 May (L. S. V. Venables, personal communication). The incubation period is about 48–57 days (Richter, 1937; Fisher and Waterston, 1941). The young fly in August, and late in that month or early in September the whole population moves seaward. In the localities from which the majority of the present

material was received (Scotland, Shetlands, Orkneys, and Bear Island) no birds usually remain about the cliffs throughout the whole year.

The material consists of testes from sixty-four fulmar petrels collected in the vicinity of nesting cliffs between January and late July. Birds taken before July were all from North British cliffs. The birds were shot and the testes dissected out and measured as soon as possible after death. As the birds were often collected in very awkward situations and dissected by voluntary helpers it was necessary to reduce fixation methods to basic simplicity. Thus, parts of each testis were fixed in formal-calcium and in Champy's fluid. Some formal-calcium material was embedded in gelatine and cut at 15μ on the freezing microtome and submitted variously to sudan black with carmalum counter-staining; or to Scharlach R. with Ehrlich's haematoxylin counter-staining for the demonstration of interstitial lipoids and the revelation of adjacent tubule contents; or to the Schultz test for cholesterol. Other formal-calcium specimens were embedded in wax, sectioned at 7μ and stained with Masson's triple stain for the determination of spermatogenetic stages, Leydig cell-counts, and the differentiation of fibroblasts from juvenile cells. The Champy material was wax-embedded, cut at 3μ , and stained with Altmann's acid fuchsin and counter-stained with brilliant cresyl blue for the study of fuchsinophil elements. All measurements in μ refer to wax-embedded material unless otherwise stated.

In the present study only an estimate of the *relative* number of interstitial cells occurring during the winter period as against those occurring in the month when sperms first appear has been made. This has been done by counting the number of Leydig nuclei in fifty fields taken from the central zone of sections of typical testes. This method shows conclusively that a considerable increase in the number of Leydig cells occurs side by side with the maturation of the tubules.

THE CYTOLOGY OF THE INTERSTITIUM

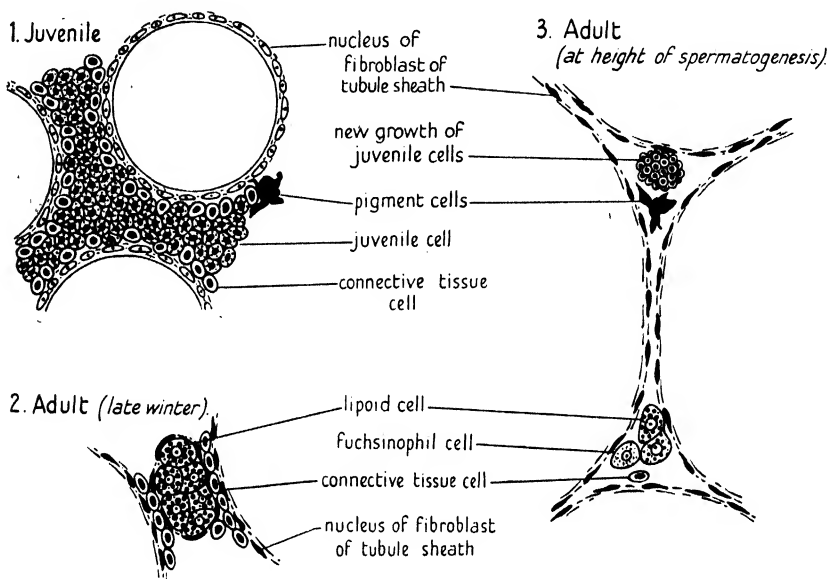
The interstitial cycle of this wild species with a clearly defined breeding season appears to be less complicated than that of the non-seasonal domestic cockerel. Sluiter and van Oordt give diagrams showing alterations in the proportions of lipid cells and non-vacuolated and vacuolated non-lipoid cells in uninjected cockerels between the ages of 2 and 200 days. In wild fulmars such striking progressive numerical variation between lipoidal and non-lipoidal cells has not been observed except during a brief annual transition-stage.

Apart from the cells present in the intertubular blood-vessels, the following cells occur in the interstitium (see diagram, Text-fig. 1):

1. Fibroblasts of the sheaths of tubules, and in relation to blood-vessels and individual groups of Leydig cells.

2. Pigment cells (dentritic cells (Becker, 1927), or melanoblasts). These vary in number from bird to bird, and in the young, or in adults when the testes are sufficiently regressed, are sometimes compacted enough to give the whole organ a dark or at least dusky appearance.

3. Juvenile cells. These are generally rounded and from 7 to 10μ in diameter (nucleus 3μ). They appear to be the sole Leydig-type present at least throughout the first year. Their cytoplasm contains lipid droplets. They become aggregated into groups but are not so markedly grouped as are the mature lipid Leydig cells into which they later develop. Their mitochondria are much less evident than those of lipid and fuchsinophil Leydig cells. In the interstitium of the young bird also occur cells of comparable size but which lack sudanophil substances.



TEXT-FIG. 1. Diagrammatic representation of the seasonal and age changes in the cell types in the interstitium of a wild bird.

4. Lipoid Leydig cells (in adult birds). These agree with the general description given by Benoit, and Sluiter and van Oordt, in the domestic cockerel. They reach a maximum size of about $20 \times 15\mu$ (nucleus $6-7\mu$), and are aggregated in groups in the sexually mature bird. By 'lipoids' are meant any of the chemically diverse fatty substances which colour with sudan black or Scharlach R.

5. Fuchsinophil Leydig cells (in adult birds). These agree with the 'glandular' phase of Benoit and the 'secretory cell A' of Sluiter and van Oordt. They are about the same size as lipid cells.

6. Areolar connective-tissue cells. These, in the young bird, may be referable to the small non-sudanophil cells mentioned under 3. In the adult bird they are quite distinct from either lipid, or fuchsinophil Leydig cell. They are non-lipoidal, and only 6μ in diameter (nucleus $4-5\mu$).

THE TESTIS CYCLE

1. Immature Birds

Odd birds taken in the vicinity of cliffs on which colonies were breeding had testes measuring from 15×10 mm. to 22×15 mm. In this species (as in many others) the left testis was almost always the larger in adults and yearling birds. During the breeding season the immature testis sometimes reached the maximum overall size, yet the tubules, containing inactive primary germ-cells, may measure only $35\text{--}40\mu$ in diameter. Comparatively few elongated fibroblast cells occur per section. Small juvenile interstitial cells are conspicuous. It has not been proved that this latter type has a different origin from the non-sudanophil circular or oval cells which appear in older birds. The juvenile cells contain varying amounts of lipid material which is dispersed unevenly through the cytoplasm in granules of varying sizes and which presents a mottled appearance. These cells contain very much less fatty substance than the large lipid cells of adults at the height of winter (January). In the birds in question no mature Leydig cells are present. No mitoses are observed. As the species has a rigidly restricted annual breeding season the above individuals cannot be less than one year old.

2. Mature Birds

January (15th to 25th). The birds have come from the sea. A big population is assembled on and around the nesting cliffs. Testes of eight birds are nearly uniform, averaging 7×4 mm. in diameter. The maximum tubule-width is 70μ . Tubule-contents are limited to spermatogonia, two cells deep, which almost occlude each tubule. The intertubular tissue exhibits groups of large lipid cells; each group is bounded by spindle-shaped connective-tissue cells. These lipid cells mostly measure about $15 \times 10\mu$ with nuclei $4\text{--}5\mu$ in diameter. The nuclei of the larger lipid cells often have two prominent nucleoli. Surrounding the groups of lipid cells are closely packed connective-tissue cells which are especially prevalent near the periphery of each testis. These smaller cells are not arranged in groups. Their diameter is limited to about 6μ and their nuclei are $4\text{--}5\mu$ thick. Treatment by sudan black or Scharlach R. brings the lipid cell-groups out in striking relief against the pale unstained tunicae propriae and tubule-contents. Pl. 1, fig. 1, shows this under low power. (Some of the tubules have been torn out by the freezing microtome, but the interstitium is not interfered with.) Pl. 1, fig. 2, shows a high-power view of individual groups in cross-section. Individual cells are so heavily laden with lipoids that a great deal of differentiation is necessary before the nucleus can be seen clearly in a 15μ section. After Champy-fixation and wax-embedding the lipoids are nearly all lost, but Altmann-staining reveals abundant fuchsinophil particles resembling mitochondria among the globular network which remains after the lipoids have dissolved. Schultz's test for cholesterol is positive in the lipid cells, proceeding so strongly as to obliterate all cellular detail. It is negative for the tubules.

Blood-vessels are very obvious in the neighbourhood of the lipid groups. No mitoses were observed. There are about 17 nuclei of lipid cells per microscopical field of view (average of 50 fields) in testes measuring $6 \times 5 \times 5$ mm.

February (first week). Seven testes average 9×6 mm. Tubules now measure up to 110μ in diameter. They still contain mostly spermatogonia with a few primary spermatocytes in synizesis. Two birds contain many spermatocytes in synizesis and up to six zones of germinal cells in a tubule. Connective-tissue cells are abundant with nuclei measuring $5 \times 4\mu$. Partial dispersion of the lipid cells, beginning at the onset of tubule expansion, can already be seen. One tract of lipid cells was squeezed out to a length of 170μ (measurement after formal-calcium fixation) in varying width. The lipid cells are highly sudanophil—it is impossible to see more than the outline of the nuclei after colouring a 15μ section with Scharlach R. After Champy-Altmann treatment the lipid cells exhibit mitochondria in the network between the empty globules. Some compressed lipid cells measure $25 \times 8\mu$ and contain nuclei $7 \times 5\mu$. Nuclei of the spindle-shaped fibroblast are now 15μ long. No fuchsinophil cells are observed. Blood-vessels are very prominent. No mitoses are seen.

February (last week). Five testes average 12×9 mm. A sharp and substantial increase in volume has occurred in the space of about a fortnight. Tubules have increased up to 150μ in diameter. All testes show maturation division and three contain spermatozoa arranged in bunches, with a few mature sperms free in the lumina. The intertubular tissue presents equally remarkable changes. Extensive empty tracts appear. The initial impression given is that hand in hand with the upsurge of spermatogenetic activity there has been a large-scale reduction in the number of Leydig cells. The intertubular tissue in a given section now generally consists of odd strands of connective tissue, prominent blood-vessels, and a few isolated groups of Leydig cells. The reason for this appearance is that the expanded tubules have dispersed the once closely packed lipid groups. Blood-vessels have become more obvious because they are no longer partially concealed by packed masses of Leydig cells and because they retain their continuity and stretch through the apparently denuded interstitium. Isolated groups of lipid cells still colour deep scarlet with Scharlach R. (Pl. 1, fig. 3). Although spermatogenesis has proceeded a long way there is no visible evidence of any transfer of lipoids to the tubules for the nourishment of the developing sperms as claimed by Stieve (1923, 1926) in the goose. Lipoidal cells are nearly all at or near maximum size. They show an abundance of fuchsinophil elements after lipid dissolution and Champy-Altmann techniques. Many connective-tissue cells are still present. Although spermatozoa have appeared, some testes are so heavily lipoidal that slides appear mottled red (after Scharlach R.) if held to the window and examined with the naked eye.

March (first week). Seven testes average 14×10 mm. Tubule contents are in much the same condition as in birds taken a week before. Although the

dispersed lipid cells turn deep scarlet or black with fat-colouring reagents, no trace of lipid is detected in the neighbouring tubules near the prominently bunched spermatozoa, though von Ebner's granules are faintly sudanophil, and give a faintly positive Schultz reaction. Lipoid cells now measure up to $20 \times 15 \mu$ with nuclei 7μ thick and nucleoli 1.5μ across. Some lipid cells still measure only $10 \times 6 \mu$, however. Areolar connective-tissue cells are also present. Counts in the largest testis ($15 \times 10 \times 10$ mm.) reveal an average of 7 nuclei per field (50 fields counted). There has been a gain of more than 300 per cent. in Leydig cells since January.

April (third week). Three testes, of which two measure 15×10 mm. and the third 24×12 mm. The two first-mentioned testes have tubules about 160μ in diameter. These contain vast numbers of mature, bunched sperms, with some free in the lumina. Lipoid cells are maximal in size and are as strongly sudanophil as in the previous months. There is no evidence of a movement of lipid into the tubules. The only tubule lipoids are the faintly sudanophil von Ebner's granules. Mitochondria show in Champy-Altmann-treated lipid cells. Maturation divisions are occurring abundantly in the tubules, but mitosis is not observed in the interstitium.

The largest testis of the group (24×12 mm.) presents a different picture. Its tubules measure up to 200μ wide. Sperms are free in enormous numbers in some lumina but are still arranged in bunches in the less-advanced peripheral tubules. Only in these outer tubules is spermatogenesis still proceeding actively in the primary and secondary spermatocyte stages. The presence of numbers of degenerating cells in some lumina shows that those tubules have passed the peak of spermatogenesis. Although there is no sign of lipoids in the tubules, the lipid content of the interstitium cells is being depleted. Lipoid cell nuclei are generally seen more readily than before after Scharlach R. colouring, and some lipid cells appear to lack sudanophil substances altogether. After Champy-Altmann techniques fuchsinophil elements show as in previous months, but two new appearances are now commonly present. These are:

1. Cells having the same general size and appearance as the largest lipid cells but lacking the characteristic globular network which appears after the lipoids are lost during embedding in wax. The nucleus is of maximal size and the nuclear cytoplasm has dark granules scattered unevenly through it. The general cell cytoplasm is speckled profusely with fuchsinophil elements which completely hide any suggestion of a globular network which may have remained. There are globular networks in adjacent cells. In one group of three lipid cells a differential mitochondrial appearance occurs. The more heavily fuchsinophil cells appear to be referable to the 'secretory cell A' of Sluiter and van Oordt. The above observations go towards substantiating Benoit's claim that lipid cells develop into cells which are heavily fuchsinophil. It must be stressed, however, that the present observations refer only to a few cells in the testis at the height of spermatogenesis.

2. There appears densely packed between two peripheral tubules a crop of

small cells very like the juvenile type. They possess nuclei about 4μ in diameter, and their scanty cytoplasm appears only faintly granular after sudan-colouring. There is at present no evidence as to the precise origin of these cells.

May (third week). The first egg of any colony under observation was seen on 13 May and a fully formed egg was dissected out of a female on the 15th. The testes of six birds of this period average 12×8.5 mm., ranging between 15 and 10 mm. in length. Tunics are now very variable in thickness. This is caused partly by the presence of voluminous blood-vessels, including veins 90μ thick—a tremendous increase in bore over those of pre-spermatogenetic months. All testes examined contained sperms but it is obvious that in the case of male fulmars the height of the season has passed. The tubules generally contain aggregations of degenerate cells and mitotic activity is mostly restricted to the extreme peripheral zones where a few spermatocytes are still dividing. The interstitium of the larger testes still contains lipid cells but fewer with the typical network after Champy-Altmann treatment. Quite often large cells are filled with a granular fuchsinophil cytoplasm instead of the former lipoids. There are several interstitial tracts filled with smaller cells of the next Leydig generation. Also noteworthy is the presence of numerous almost empty tracts measuring up to $40 \times 150\mu$ which follow the contours of the shrinking tubules. Blood-vessels, connective-tissue fibres, and rounded or oval fibroblasts pass across them.

June. Three testes average 10×6 mm. Tubules are now 110μ in diameter. This is a period of profound testis regression and reorganization. In the tubules a remarkable new epithelium has appeared. This is at first composed of a single layer of large cells with nuclei 5μ in diameter. In some tubules two layers have appeared and altogether the epithelium may be 30μ thick. Isolated in the centre of many of the testis lumina are masses of degenerate germ cells up to 35μ thick, and some lumina are completely choked with these degenerating products of the former spermatogenesis. In some testes, however, some tubules are already almost free of debris.

The tubules of the testis have meanwhile become lipoidal and react positively to Schultz's test. The degenerating cells interior to the new epithelial generation are heavily sudanophil and the sloughed degenerating material somewhat less so. The new basal layer of epithelial cells is sudanophil to a varying degree. In some testes the cells are heavily lipoidal near their free borders; in others only a meagrely sudanophil granular mottling is retained.

The interstitium too is in a regenerative phase. The former generation of Leydig cells has disappeared and the interstitium is filled with a new generation which resembles the juvenile cell and which first made its appearance in the largest testes of late April at a period when fuchsinophil cells first became plentiful and the most-advanced tubules exhibited signs of epithelial degeneration. The smallest cells between the tubules in June are only faintly lipoidal. At odd places in the testis they still remain aggregated together in tracts

100–200 μ long. In these tracts of undispersed new Leydig cells there also occur cells which are larger and more heavily lipoidal, and which measure 10 \times 6 μ with nuclei 4 μ in diameter. These are not organized into groups. Elsewhere in the interstitium the lipoid Leydig cells have reached maximal size and are grouped. The groups are bounded by spindle-shaped fibroblasts. After Champy fixation and Altmann staining, numerous minute mitochondria are observed. These very closely resemble Sluiter and van Oordt's fuchsinophil type. The interstitium also contains pigment cells. Arterioles 30 μ in diameter, with a bore of about 15 μ , and veins of the same volume, also transect the interstitium.

Late July (15 specimens). Testes average 7 \times 5 mm. These birds were collected on Jan Mayen and Bear Islands in the Arctic during 1947 and 1948. They were taken in the vicinity of the nesting cliffs after the young were hatched. Each bird used in the present study showed a 'brood patch' which probably indicates that it has reproduced (in *Fulmaris* the sexes take turns at incubating the eggs). Each bird was now moulting. These testes present a uniform picture except that the new tubule-epithelium, consisting of two layers of spermatogonia, is slightly uneven in its sudanophil properties. No tubule, however, retains sufficient lipoids to exhibit a large vacuole after wax-embedding. Tubules are regressed to 50 μ and are occluded by the new germinal generation; degenerated remnants of the last spermatogenesis have been entirely eliminated. The interstitium is more or less heavily lipoidal in all birds, with the Leydig cells generally organized into groups which are dispersed evenly. Several testes, however, show tracts of meagrely sudanophil small Leydig cells apparently in the process of becoming lipoidal. These are still retained undispersed in restricted areas. The mature Leydig cells, and many of the smaller ones, show mitochondria. Pigment cells measure about 20 \times 5 μ and their nuclei may be seen without bleaching in Champy-fixed and Altmann-stained material.

Both the tubules and the interstitium are now almost completely regenerated (Pl. 1, fig. 4).

Late July (2 specimens). Non-breeding birds with no brood patch. These reveal a testis-type which does not fit into any group so far described. Testes measured 6 \times 4 mm. and those of both birds were dark with concentrated pigmentation. The tubules were 70 μ wide and contained primary spermatocytes.

Had it not been for their curious interstitium these birds would have passed for winter adults. The interstitial cells were mostly of the juvenile type measuring 7 \times 5 μ with nuclei 5 \times 4 μ thick. While the nuclei were still small some of these cells had become more strongly sudanophil, and many, in the centres of the wide interstitial tracts, were now approaching adult size (24 \times 10 μ with nuclei 6 μ wide). Each testis gave the impression that more and more juvenile cells were becoming heavily lipoidal until big groups of almost mature Leydig cells, bounded by spindle-shaped connective-tissue cells, had appeared (Pl. 1, fig. 5).

The above testis is nothing like that of the July adult, and quite unlike that of the yearling bird. A comparable appearance has been observed (Marshall and Coombs, unpublished) in the rook at a period when the 16-months-old young was changing by moult to its first adult plumage. (The age is known from details of plumage.) As the testis of the fulmar yearling presents an entirely different picture, it may be that the present birds are just over two years old and will breed the following year.

DISCUSSION

In sexually immature fulmars a small partially lipoidal *juvenile* cell occurs in the intertubular tissue of the testis. This cell develops into the large and strongly sudanophil mature *lipoid* Leydig cell not before one year and possibly when the bird is just over two years old. Once sexual maturity is reached, the bulk of the Leydig cells appear to belong to the lipoid type throughout the year except for a brief period which begins about the height of spermatogenesis. From the time the sperms have begun to appear, the Leydig cells are seen to be losing their lipoids until only a faint mottling of sudanophil substances remains in the cytoplasm. At this period numbers of non-lipoidal or partially lipoidal and strongly fuchsinophil Leydig cells appear. At the stage when free sperms are visible in the tubules and degenerating epithelial products begin to appear, a Leydig cell regeneration begins at odd spots in the interstitium. At the time of testis collapse and an accompanying fatty metamorphosis of the tubular epithelium, there appears a massive new development of small Leydig cells which may be identical with the juvenile cell in the young bird. These soon become partially lipoidal. They become distributed through the denuded interstitium, gain in size and sudanophil content, become organized into groups bounded by fibrous connective tissue, and so the interstitial cycle has begun once more. The testis is still at its minimal post-nuptial size and apparently in the 'inactive' phase (of various authors) unless the interstitium is studied with appropriate fat-colouring reagents. Meanwhile, under the fatty metamorphosis, the tubule-epithelium has become regenerated and a curiously uniform new generation of epithelial cells has been laid down. These are now meagrely sudanophil only near their free borders. The tubules, too, are now practically free of debris from the former spermatogenesis and the testis is apparently completely rehabilitated. The young of the current breeding season are still in the nests. An anatomical basis for an internal rhythm may thus have been shown. It will be recalled that Bissonnette and Wadlund (1931) report that their light-stimulated starlings could only maintain spermatogenesis for a certain period after which regression began.

While the above cycle is proceeding (when the Leydig cells are predominantly lipoidal), the fulmar population has come in from the sea and is established in vast numbers on its breeding cliffs in December. This movement to the breeding territory occurs while daylight is decreasing. Between January (when its Leydig cells appear to be uniformly lipoidal and when the

testes average only 7×4 mm.) and mid-May (when the first eggs are laid) the birds take up territory, perform sexual display, select egg-sites, and follow the usual pattern of pre-nuptial behaviour which is generally considered to be under neuro-humoral control (Marshall, 1936). Meanwhile spermatogenesis is completed and copulation occurs. It is necessary to attempt to relate these factors, and to try to bring the experimental work of Sluiter and van Oordt and the results of other workers into line with the cycle outlined above.

In *Fulmaris* the testis measures about 15×10 mm. in early March, when free sperms have appeared in the lumina. Although the testis is still far below its maximal size its volume has increased greatly. With this overall enlargement there is a decrease in the thickness of the testis tunic and a considerable increase in the diameter of the tubules, which accommodate the developing germinal elements. As the tubules expand the tightly packed Leydig cells disperse, creating an appearance, in any given section, of a general decrease in interstitial cells at the time of spermatogenesis. This is a false appearance: lipid Leydig cells increase by at least 300 per cent. between mid-winter and the time of spermatogenesis. The present study therefore agrees with those of Benoit (1929), Groome (1933), and Sluiter and van Oordt (1947), who state that the Leydig cells of birds increase in number with heightened sexuality.

The *Fulmaris* material makes it clear that in the interstitium of young birds there occurs a meagrely lipoidal cell which develops into a mature lipoidal Leydig cell, a process also noticed by Benoit. It is the next stage in the cycle which remains still in doubt, and the matter is inextricably bound up with the question of the relative function of the lipid and fuchsinophil Leydig-cell types. After sperms have appeared free in the tubule lumina, most Leydig cells are still too densely sudanophil to show even the nucleus clearly in a 15μ section. From now onwards, however, a steady diminution of the lipid content takes place. At the time when the testes are about to collapse, most of the lipid cells show only granular sudanophil substances in their cytoplasm, and even taking tubule-expansion into consideration it is evident that their numbers have decreased. Stieve (1926) noted the decrease in lipid content in Leydig cells and published a coloured plate (p. 15 of the above reference) illustrating his theory that Leydig cells are trophic in function and give up lipoids to nourish the developing germinal epithelium. Occasionally during the present investigation frozen sections coloured with Scharlach R. appeared to agree with Stieve's illustration in that they showed a scarlet mottling appearing dispersed among the spermatogenetic products. On repetition, however, it was found that the above appearance, in *Fulmaris*, had been caused by errors in technique; interstitial cells had been ruptured and small coloured lipid droplets had become lodged in the neighbouring tubules of the section. No sudanophil substance was detected in the tubules until mature sperms appeared. At this stage von Ebner's granules exhibit a lipophanerosis and are faintly but unmistakably lipoidal.

Benoit declared that the lipid cell loses its sudanophil contents and develops into a fuchsinophil cell. Sluiter and van Oordt failed to confirm this transition, and left the question open. In *Fulmaris* the lipid cell (after Champy-Altmann techniques) often shows abundant mitochondria among the network between empty vacuoles from which the lipoids have dissolved. At about the height of spermatogenesis almost identical cells occur immediately adjacent to the lipid cells; these differ only by the possession of a more profuse development of mitochondria. It is believed then that Benoit's view was correct. Benoit did not observe Sluiter and van Oordt's vacuolated fuchsinophil cell ('secretory cell B'), into which the latter authors believe the non-vacuolated fuchsinophil cell develops. This 'cell B' of Sluiter and van Oordt has not been observed in *Fulmaris* material, but something like it has been seen by the present author in other species.

Sluiter and van Oordt declare that the vacuolated fuchsinophil cells 'undergo a regression and pass over into lipid cells'. Of this, no confirmation has been found in *Fulmaris*. Between the two generations of Leydig cells intervenes a new appearance of small meagrely lipoidal cells which spring up at odd points of the interstitium after the most-advanced tubules have shed sperms and while the less-advanced tubules still contain dividing spermatocytes. This new Leydig cell generation appears abundantly during the period of tubule breakdown. A big tract of small cells shows only a faint tinge of sudan colouring when they are very young and so for a time the interstitium is almost totally devoid of lipoids. At this stage, however, fatty metamorphosis is proceeding in the tubules. The tubules become brilliantly sudanophil. Rowan (1929) has evidence of this tubule-metamorphosis, describing tubal 'vacuities'. Rowan mentioned that the 'vacuities' look as though they had been artificially produced, but emphasizes that they are not artifacts. He was right; they are the empty spaces left after the fats were dissolved out during alcohol grading. Bissonnette (1930) also mentioned the 'vacuolation' of the epithelium in the regressive testis, apparently without realizing its significance.

It is suspected that the new Leydig generation arose from the old generation of exhausted Leydig cells but no proof of this can be offered.

The present work does not substantiate the opinion of Rowan and Batrawi (1939) that the winter interstitium consists solely of spindle-shaped connective-tissue cells with heavily staining nuclei, nor can any confirmation be offered of Bissonnette and Chapnick's (1930) suggestion that the interstitial cells may disappear in the spring by being incorporated in the tunicae propriae as they distend to accommodate the ripening tubule-products.

We now come to the question of the relative function of the two cells: lipid and fuchsinophil. Sluiter and van Oordt injected cockerels with gonadotrophin obtained from pregnant mares' serum and attempted to equate the resultant accelerated growth of the birds' head-appendages with the cytology of the interstitium. They report that the total number of all three interstitial cell-types (lipoid, fuchsinophil, and vacuolated fuchsinophil) increased enormously as the head-appendages developed. In normal, uninjected birds

about 50 per cent. of this increase in number is due to the increase in lipid cells. In birds treated with gonadotrophin only the fuchsinophil cells increased in number. Sluiter and van Oordt therefore term these 'secretory cells'. Because the head-appendages of gonadotrophin-treated birds increase in size along with the increase of non-lipoid cells, the above authors declare that it is obvious that the lipid cells are not directly necessary for the production of hormone. They showed that the lipid cells contain cholesterol derivatives, but believe that although the lipid cells cannot be directly concerned with the production of androgens they may act as storage cells of 'special elementary substances'.

In *Fulmaris* wherever the Leydig cell is positive to sudan black or Scharlach R. it gives a positive Schultz cholesterol reaction. Later in the season, when the lipid cells have lost much of their sudanophil content, only a faint blue-green mottling appears when the Schultz test is applied. It has not been possible to locate a non-lipoidal fuchsinophil cell in any section (i.e. formal-calcium-fixed) to which Schultz test can be applied.

In other parts of the testis the rule holds: if a section of tissue colours with Scharlach R. then it will be Schultz positive. This extends to the metamorphosing tubules, von Ebner's granules, and the regenerating epithelium of the vasa efferentia after the breeding season.

Although the biochemistry of the sex hormones *in vivo* is still obscure, their derivation from cholesterol in nature appears to be a comparatively simple process. Like the present investigator, Sluiter and van Oordt established the presence of the probable precursor of the sex hormone in their 'storage' cells but were not able to do so with certainty in their 'secretory' cells.

Apart from its richness in cholesterol, the present author believes that the lipid phase of the Leydig cell is the primary producer of sex hormone. Lipoid cells increase in number during heightening sexuality in the wild bird, and are the only apparently active cell-type present during the period when the birds flock back to their nesting cliffs, take up territory, display to each other, take up nest-sites, and achieve spermatogenesis. With the appearance of abundant spermatozoa, completely fuchsinophil cells appear in quantities, and it is not denied that they may possess some glandular significance. The present author, however, inclines to the idea that they are merely a phase of the endocrine lipid cell and that Sluiter and van Oordt's injections perhaps stimulated and accelerated the naturally slow evolution from juvenile→lipoid→fuchsinophil cell. It could be that the appendage growth in the cockerels came from the stimulated lipid cells, which, having discharged their endocrine function, passed over to the fuchsinophil phase.

Sluiter and van Oordt, while not agreeing with Stieve (1923, 1926) that the lipid cells are solely trophic in function, consider them primarily storage-cells and state that their number depends on the nutritive conditions of the animal. In *Fulmaris* there is no evidence that this is so. Again, Sluiter and van Oordt suggest that lipoids sometimes occur in such quantities as to leave no room for a glandular function in the cell. Wells (1925), however, has

evidence that the liver cells of man (especially among alcoholics) may sometimes contain so much fat it is difficult to find any cell cytoplasm, yet there is no clinical evidence of any impairment of function.

Sluiter and van Oordt state that many vacuolated fuchsinophil cells in cockerels older than 2 months 'undergo a regression and pass over into lipid cells' and that in the adult cock 'only a small percentage of the original secretory cells still function as such . . . the others have become storage cells'. In *Fulmaris* a new meagrely lipoidal generation, described above, comes after the advent of fuchsinophil cells and before the next appearance of large lipid Leydig cells. The only way in which we can bring the above opinion of Sluiter and van Oordt into line with the present work is to think of their mitochondrial 'secretory' cells as the new generation of partially lipoidal Leydig cells which do, in fact, also exhibit mitochondria. In any case, the gaining of lipoids, far from being a 'regression', is actually another active phase in the cycle. The interstitium, in short, is once more becoming lipoidal in preparation for the forthcoming activity connected with the next breeding cycle.

ACKNOWLEDGEMENTS

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SUMMARY

1. An investigation of the seasonal cycle of the interstitium of the testes of birds, based on sixty-four individuals of varying age, has been carried out on the fulmar petrel, *Fulmaris glacialis* (L.).

2. The following cells (see diagram, p. 268) occur:

- (i) Fibroblasts of the sheaths of tubules, blood-vessels, and of groups of Leydig cells.
- (ii) Pigment cells.
- (iii) Juvenile Leydig cells (generally containing lipid droplets).
- (iv) Lipoid Leydig cells.
- (v) Fuchsinophil Leydig cells.
- (vi) Areolar connective-tissue cells.

3. In the young bird the juvenile cell develops into the lipid Leydig cell at a time when the testis-tubules also indicate approaching sexual maturity.

In the adult, the interstitium generally consists for the most part of lipid Leydig cells. These exhibit mitochondria when the soluble lipoids are removed by embedding in wax. At the height of spermatogenesis, and at the beginning of epithelial breakdown and tubule-regression, most of the Leydig cells have lost much of their lipoids and some of them exhibit increasing amounts of fuchsinophil substances, as described by Benoit. A fuchsinophil cell thus appears.

4. The Lipoid cell at all seasons shows a positive Schultz reaction (for cholesterol), which corresponds in intensity to the amount of sudanophil material present. It has not been possible to demonstrate cholesterol in the fuchsinophil cell.

5. At the time when the tubules are at their maximum diameter and their degeneration is under way, mitochondria are at the greatest abundance in the Leydig cell. At this period a new generation of Leydig cells arises in the interstitium. These cells quickly become meagrely sudanophil and resemble the sudanophil juvenile cells of the immature bird. They are Schultz positive. The tubules collapse; the new Leydig generation fills the empty interstitium. They exhibit profuse mitochondria, gain in lipid content and so the interstitium is regenerated.

6. The exhausted tubules undergo a fatty metamorphosis at the time of their collapse; at the period when the interstitium has little lipid the tubules are full of it. Beneath this fat (also Schultz positive) arises a new tubule-epithelium. The tubules as well as the interstitium are regenerated while the young of the next generation are still in the nests. An anatomical basis for an internal physiological rhythm may thus have been shown.

7. The new interstitial cells are already heavily lipoidal and the new tubule epithelium contains spermatogonia when the petrels move seaward away from the breeding cliffs in the autumn. They return to the breeding area in November and December. Thus testis regeneration, and movement both away from and back to the breeding area, occurs while the days are getting shorter.

8. From the time the birds appear on the breeding cliffs (and apparently since the autumn) there is an increase in lipid cells along with heightened sexuality as revealed by the ripening tubule-products. These supplementary lipid Leydig cells seem to develop from the small non-sudanophil areolar connective-tissue cells which are prominent in the adult interstitium.

9. Whilst it is not denied that the fuchsinophil cell ('secretory cell' of Sluiter and van Oordt) may have an endocrine function, the present results suggest that the lipid Leydig cell is the primary secretory component of the avian testis. When the interstitium, after reproduction, passes once more to a lipoidal phase, it is not losing its secretory function as Sluiter and van Oordt infer, but is regenerating in readiness for the next season's breeding activities.

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EXPLANATION OF PLATE I

- FIG. 1. Adult winter testis (January). Low power, showing A. Lipoidal interstitium; B. Tubules free of lipoids; C. Tunica albuginea. (Sudan black and carmalum at 15 μ .)
 FIG. 2. Ditto. High power, showing A. Individual group of lipoid Leydig cells; B. Tubule containing spermatogonia. Top tubule has been removed by freezing microtome. (Sudan black and carmalum at 15 μ .)
 FIG. 3. Adult pre-nuptial testis (late February) showing A. Group of lipoid Leydig cells; B. Pigment cell; and C. Spermatozoa. (Scharlach R. and Ehrlich's haematoxylin at 15 μ .)
 FIG. 4. Adult post-nuptial testis (late July) showing A. Regenerating lipoidal interstitium; and B. Last remaining lipoids of fatty metamorphosis in tubules. (Scharlach R. and Ehrlich's haematoxylin at 15 μ .)
 FIG. 5. Change-over phase (late July) from juvenile to mature lipoid Leydig cells. A. Indicates group of cells which have become large and lipoidal; B. Shows undifferentiated juvenile cells. (Sudan black at 10 μ .)

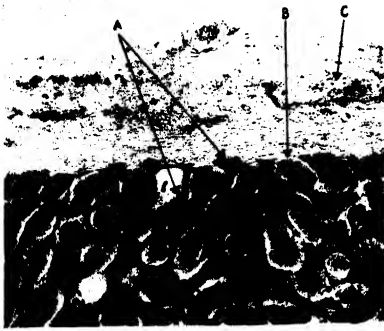


FIG. 1

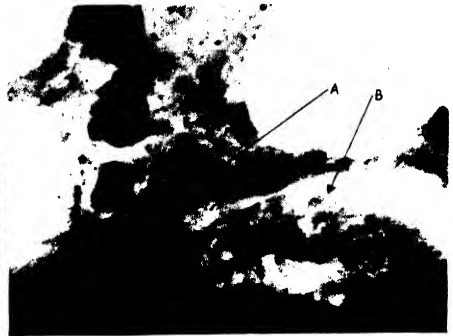


FIG. 2

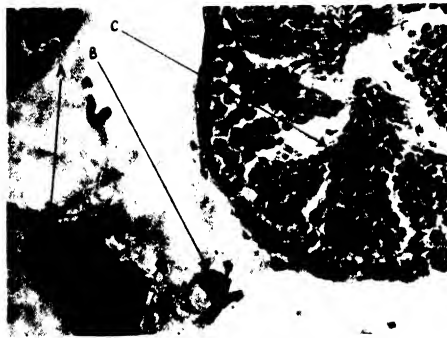


FIG. 3

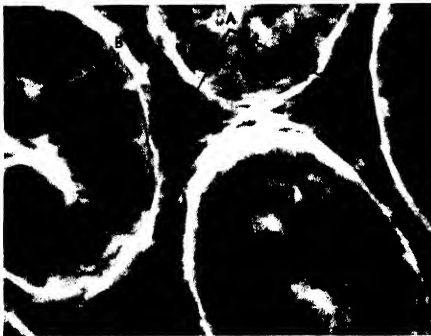


FIG. 4



FIG. 5

The Mechanism of Insemination and the Mode of Action of the Spermatophore in *Gryllus domesticus*

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INTRODUCTION

THE literature on the different types of spermatophores in Orthoptera is voluminous, yet detailed accounts of their function are lacking. Spann (1934) showed that the transference of sperm enclosed in spermatophores was observed by the early writers such as Siebold and by Lespes (1855). Since then descriptive accounts have been published by a number of authors, the most important of which are Boldyrev (1912-14) and Gerhardt (1913-21).

Regen (1924) working with *Liogryllus campestris* brought to light the mechanism by which a spermatophore is evacuated. His description cannot, however, be applied to *G. domesticus*. This might be attributed either to structural variation or to his having overlooked certain details which would necessitate some modifications based on the study of the house cricket spermatophore.

G. domesticus L. has proved to be ideal material for this study. A couple can be brought to copulate in a limited space and a young male can produce, on a hot day, two or three spermatophores.

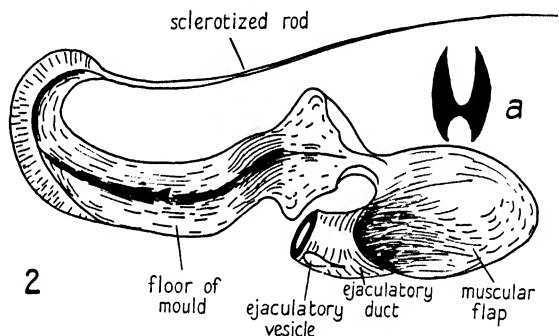
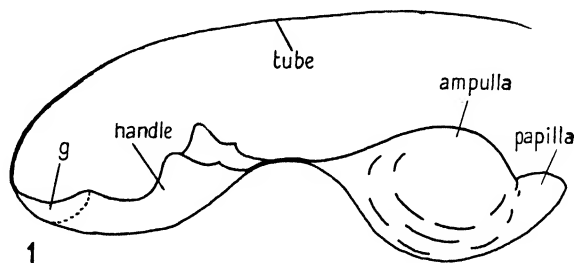
THE STRUCTURE OF THE SPERMATOPHORE

The spermatophore of *G. domesticus* (Text-fig. 1) is composed externally of three parts: the ampulla, the handle, and the spermatophore tube. When the spermatophore is in the mould, which is at the hind end of the male's body, the ampulla is found lying on the last sternum covered on each side by a lateral muscular flap (Text-fig. 2). The handle extends forward, fitting into the floor of the spermatophore mould. The tube, passing through the handle, bends on itself and extends posteriorly, lying in a very thin sclerotized grooved rod (Text-fig. 2). The convex surface of the handle will, therefore, mark the ventral surface of the spermatophore.

The ampulla (Text-fig. 1). This is a globular structure with a teat-like projection at its posterior end, the papilla. It varies in colour and consistency according to how long it has been ejected. When it is first ejected it has a white milky appearance and a very soft viscous consistency. About one hour later it becomes glassy, and a brown colour appears at the posterior end and extends a little way down the sides along the inner surface of the inner layer (Text-fig. 3d). It then becomes very hard and brittle.

The wall consists of four clearly distinguishable layers (Text-fig. 3).

1. The outer layer is a thin transparent membrane becoming comparatively thick at the posterior end of the ampulla and round the papilla. In a recently ejected spermatophore it is a smooth membrane and gives the ampulla a



TEXT-FIG. 1. The spermatophore of *Gryllus domesticus*. *g*, gelatinous white material covering the invagination at the end of the spermatophore handle (marked with a dotted line).

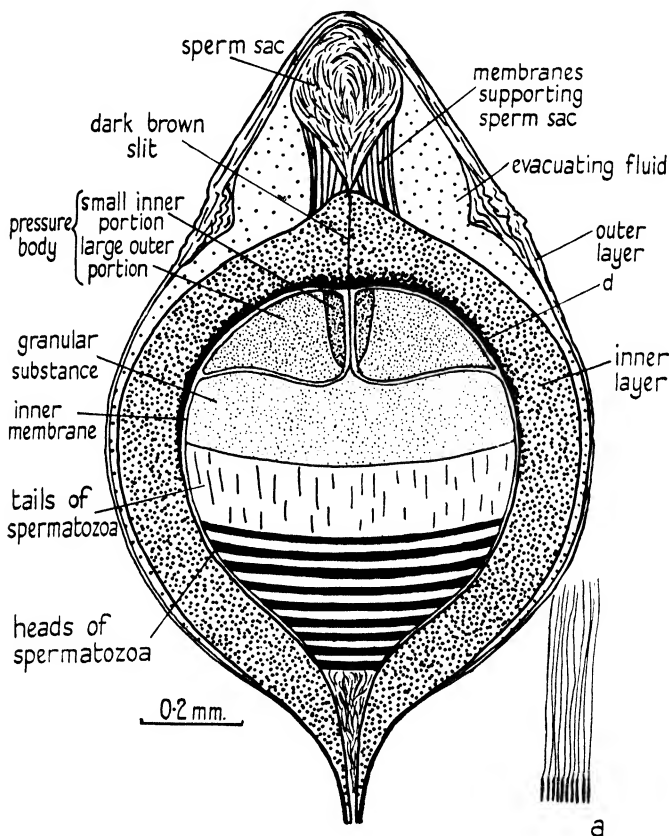
TEXT-FIG. 2. The mould of the spermatophore. The second muscular flap has been removed. *a*, a transverse section through the sclerotized rod.

spherical shape. In an empty spermatophore, however, or in one that has been kept in the mould for a fairly long time in dry air, the membrane is no longer smooth but shows many folds as it collapses on the inner layer. In sections it appears to be composed of several lamellae.

2. The evacuating fluid fills the gap between the outer and the inner layer. This gap widens posteriorly where the fluid increases in quantity, and narrows anteriorly as the outer and the inner layers approximate. When a smear of this fluid was left to dry on a slide a very thin transparent film remained. When a drop of Millon's reagent mixed with glycerine was added and the slide heated a very faint red colour appeared. The ninhydrin reaction was also positive; so it is most probable that this fluid contains protein. At a rough estimate 0.001 c.c. of this fluid is present round the ampulla. In an empty

spermatophore or in one which has been left to dry, the fluid disappears, the outer layer collapses and the ampulla loses its even surface.

3. The inner layer is the most conspicuous layer owing to its hardness and thickness (about 0.17 mm.). It is very firm and brittle, and maintains its



TEXT-FIG. 3. A horizontal section through the spermatophore ampulla. *d*, the dark-brown colour extending along the inner surface of the inner layer; *a*, a diagrammatic representation showing the arrangement of the spermatozoa.

shape in a filled or empty spermatophore. The hardening of the ampulla is entirely attributable to certain chemical changes taking place in this layer as the ampulla is exposed to the air. A newly ejected spermatophore could be squeezed between a slide and a coverslip, and the granular nature of this layer could be easily seen. Later, however, it becomes a hard homogeneous transparent structure, and the brown colour appears. At the posterior end a

minute dark-brown slit traverses a small projection of this layer into the papilla. Removal of the papilla, thus exposing the slit, shows that the opening must be blocked, and the contents of the ampulla and papilla absolutely isolated.

4. The inner membrane is a very thin transparent structure lining most of the inner layer. It takes origin from the posterior end of the inner layer, passing in between two adjacent symmetrical thick bodies (the pressure body of Regen) and is attached to the inner layer at the other end of the capsule near the origin of the tube. It is also attached to the same layer all down the walls of the ampulla along its sagittal plane, but loose elsewhere. The inner membrane extends through the posterior part of the inner layer forming another sac in the papilla.

THE CONTENTS OF THE SPERMATOPHORE CAPSULE

1. The pressure body is a translucent mass of thick consistency divided up into two symmetrical parts, each surrounded by a separate transparent membrane and enclosed, at the posterior end of the spermatophore capsule, between the thick inner layer and the inner membrane. In cleared preparations and in sections, each half of the pressure body can be differentiated into a smaller inner and a larger outer part. The two parts show different staining properties but both give protein-positive reactions.

2. A granular substance is found underneath the pressure body inside the capsule surrounded by the inner membrane. It is a viscous fluid, and when examined under the microscope in a drop of water its fine granules show Brownian movement. It is termed by Spann (1934) the seminal fluid and she presumes that it has a nutritive function, since it accompanies the sperm in the spermatheca.

3. The sperm mass occupies the remaining part of the capsule. The heads of the spermatozoa are closely packed in 8–10 layers and directed towards the opening of the spermatophore. Their long tails have a silvery shining appearance, and are packed together below the granular substance. The spermatozoa are immobile in the spermatophore.

The papilla (Text-fig. 1). This is a peculiar structure at the posterior end of the ampulla. It contains a small sperm sac formed by the extension of the inner membrane. The sperm sac is supported by a considerable number of thin membranes (Text-fig. 3) that take origin from the inner layer. As has been stated, the contents of the papilla are completely isolated from those of the ampulla and so never have access to the female organs.

The ampulla varies in size from one insect to another, but it seems that every male produces spermatophores of the same size. The ampulla is generally 1.5–1.7 mm. in length, including the papilla, and 1–1.3 mm. wide in the widest part.

The handle (Text-fig. 1). This is a continuation of the outer layer. It is separated from the ampulla by a short neck, and has two lateral extensions each with a pointed process. At the other end there is an invagination which

fits tightly over the pointed end of the spermathecal spout of the female. In a spermatophore detached from its mould the invagination is covered by a gelatinous substance (Text-fig. 1g). The handle was called by Regen 'the fixing apparatus' because it fixes the spermatophore in the genital opening of the female. The two lateral extensions fit exactly on the base of the ovipositor from the ventral side, and having the end of the spermathecal spout fitted in the invagination the spermatophore is carried safely by the female for a considerable length of time. The dimensions of the handle are about 1.5 mm. in length and 1.25 mm. in width when the two lateral processes are fully extended.

The spermatophore tube (Text-fig. 1). This is an extraordinarily fine capillary tube originating from the inner layer. It extends through the middle of the handle and about 3.65 mm. beyond it. It gradually tapers towards the end where it terminates in an extremely fine-pointed closure. It has a double wall and is dark brown in colour.

It has been presumed by Jensen (1911), Spann (1934), and others that the spermatophores are chitinous structures. This has been found to have no foundation, since a spermatophore dissolves completely in a hot saturated solution of caustic potash. Millon's and the xanthoproteic tests as well as the ninhydrin reaction prove that it is built up of protein. Fat tests give negative results.

THE PROCESS OF SPERMATOPHORE EVACUATION

The mechanism of sperm transference is in most insects a problem that has not been satisfactorily solved. This is perhaps due to the meagre amount of secretions found in the different parts of the sexual tract, which has made investigation of their chemical composition and physical properties difficult. In the cricket spermatophore the migration of sperm to the spermatheca is carried out mainly if not entirely by forces provided by the ampulla which remains outside the female organs after copulation. The problem is here, therefore, much simpler than it is in those insects, e.g. *Lepidoptera*, where spermatophores are introduced inside the female bursa, and where sperm, stimulated, activated, and directed by unknown factors, leave the spermatophore and take a most complicated path to the spermatheca.

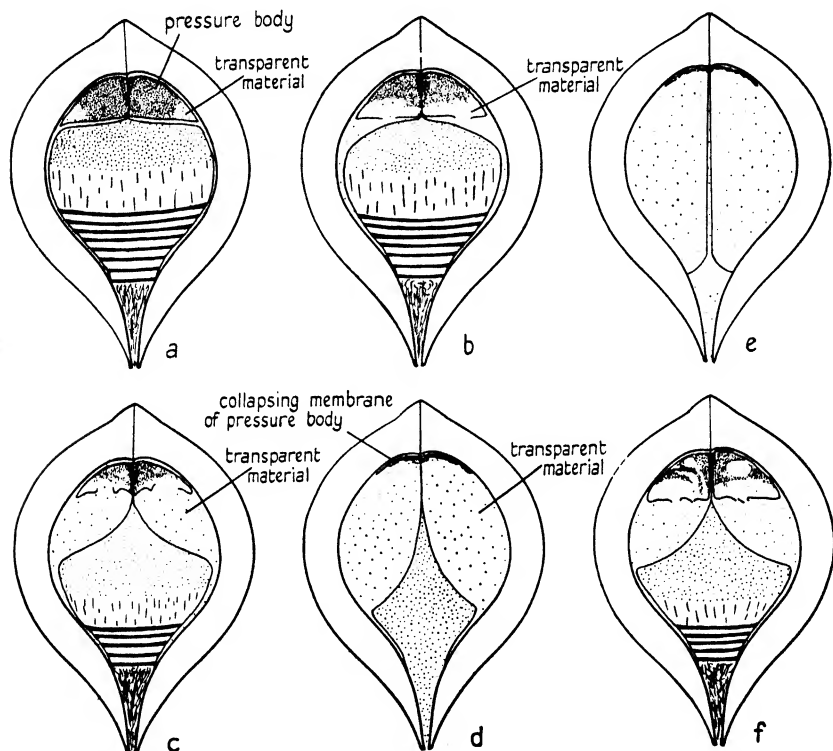
Regen's explanation. Regen has shown that the fluid enclosed between the outer and the inner layer is of great importance in emptying a spermatophore. As long as the spermatophore is kept in its mould the tube remains closed and the contents of the ampulla have no access to the outside. When the spermatophore was examined in water he could see and remove the closure, so that the sperm started to come out. He presumed that when the spermatophore tube was inserted into the spermathecal duct during copulation the closure would dissolve in the spermathecal duct to allow the sperm to flow out. When the tube was broken on a slide a flow of sperm would also start to come out and would continue for some time, until no fluid remained between the two layers of the ampulla. This also happened when a spermatophore

was delivered to a female. When a spermatophore was deprived of this fluid and the tube was broken nothing came out, but when a spermatophore so treated was put in water the flow started. Inside the ampulla, on the other hand, he found the pressure body underwent a swelling process as the spermatophore emptied itself. So a pressure was exerted inside the ampulla, and as the size of the capsule remained constant because of the great resistance of the inner layer all the contents were forced out. The pressure, he deduced, originated when the outer fluid penetrated the inner layer (after the closure at the end of the tube had been removed) and caused the pressure body to swell. He also stated that the pressure body was also pushed to the outside by the pressure it generated on itself.

Description of the process of evacuation. The different stages in the process of emptying a spermatophore were worked out by watching the process under a binocular microscope. When a spermatophore was placed on a slide and had its tube cut somewhere between the handle and the ampulla, the flow of sperm would continue for about 19 minutes. This was followed by the flow of the granular substance, which took about 15 minutes. To make observation easier the ampulla was deprived of the outer layer, which could be easily done when the spermatophore was still in the mould, and dipped in water. When the tube was broken at the above-mentioned point the outflow of sperm started and took about 11 minutes. To cause the process to extend over a long time so that the different stages could be clearly observed, concentrated salt solutions were used instead of water (see Table 1 on p. 289).

Two small transparent areas (Text-fig. 4a) first appear on both sides of the ampulla at the periphery of the pressure body. The transparent material increases gradually in quantity inside the two membranes surrounding the two halves of the pressure body; eventually a stage is reached where the two membranes cannot resist the increase in size and burst (Text-fig. 4b). The transparent material now occupies the space between the inner layer and the inner membrane on each side of the ampulla. As this material increases in quantity the inner membrane is pushed inwards (Text-fig. 4c and d) and a pressure is exerted on the contents of the ampulla, causing them to flow out. The contents of the ampulla continue to come out until the translucent pressure body has become entirely transparent. When an intact spermatophore was evacuated on a slide this stage was reached while a certain amount of the granular substance was still in the ampulla (Text-fig. 4d). This also occurs with spermatophores delivered to females. In other words complete evacuation is never obtained with spermatophores delivered to females or emptied on a slide. In water or dilute salt solutions, however, the sperm as well as the whole amount of the granular substance come out. In such empty spermatophores the inner membrane, which is at the beginning a smooth stretched structure lining the inner layer, is now folded and takes a vertical position along the sagittal plane of the spermatophore, standing in the ampulla as a double layer partition (Text-fig. 4e). In some cases, however, the inner membrane is seen to lose contact with the inner layer along the

sagittal line, producing a continuous space surrounding the collapsed inner membrane which is occupied by the transparent material. The two membranes which surround the two halves of the pressure body appear at the end as two collapsed structures at the top of the capsule (Text-fig. 4*d*).



TEXT-FIG. 4*a, b, c, and d*. Four stages in evacuating a spermatophore on a slide by cutting the spermatophore tube somewhere between the ampulla and the handle. These stages represent what happens in the natural condition. *e*, Shows the complete evacuation when stage *d* was put in water. *f*, Shows the result of soaking a spermatophore—which had had the outer layer removed and the evacuating fluid dried up—in 1.5 M sucrose solution for 12 hours. It shows the ampulla as partly evacuated.

In all diagrams the outer layer and the evacuating fluid are not represented.

Role of osmosis. The fact that the evacuating fluid has great importance in emptying the spermatophore and that this fluid diffuses inward when the closure is removed, is true of the spermatophores of both species. A spermatophore that was kept in its mould or on a slide for such a long time in dry air that the evacuating fluid had dried up, was no longer functional; when the tube was broken nothing came out. The outer layer in such spermatophores is folded like that of an empty ampulla. A drop of water, or any dilute salt solution on the ampulla acts like its normal fluid and the sperm starts to come

out. The ampulla is normally protected against desiccation by the two muscular flaps almost covering it, which contain a considerable amount of body fluid.

The outer layer, beyond enclosing the evacuating fluid round the ampulla, has no other function so far as the process of emptying the spermatophore is concerned. An ampulla that had this layer removed but still retained traces of the evacuating fluid worked normally when the spermatophore tube was broken. Moreover, an intact ampulla which was left for a while in dry air to get rid of the evacuating fluid still worked normally when dipped in water or any dilute salt solution. The outer layer is, therefore, acting as a permeable membrane, and has no mechanical or physiological effect on the penetration of the evacuating fluid through the inner layer.

Regen stated that when a spermatophore was dipped in water nothing came out unless the closing plug was removed. In the present work, however, in water as well as dilute salt solutions the spermatophore tube sometimes swelled and burst at some points along its length, and so the sperm had access to the outside. In some other cases where this did not occur, and the spontaneous removal of the closure also failed, the thick inner layer cracked and all the contents came out. This shows that water is more efficient in emptying a spermatophore than the natural fluid surrounding the ampulla. This is evident from the fact that the whole amount of sperm comes out in a shorter time when water is used in evacuating the spermatophore than when it is evacuated on a slide (11 against 19 minutes). It follows that the evacuating fluid must have a certain limited osmotic pressure, thereby avoiding rupture of the tube or of the ampulla as long as the spermatophore is kept sealed, as in the mould.

Many spermatophores were deprived of the outer layer of the ampulla, taken away from their moulds and left on a slide for some time to dry. Bits of filter-paper were also used to remove as quickly as possible all traces of the evacuating fluid. When the ampulla became completely dry the spermatophore tube was cut somewhere between the ampulla and the handle, and the ampulla was dipped in a small amount of salt solution in a watch-glass. Every such treated spermatophore was used once only. The results of a typical experiment repeated a number of times are shown in Table 1. No explanation is offered of the curious order of relative effectiveness of the salts, but this order was found to occur in every case.

It is evident, therefore, that with MgSO_4 , KCl , and KNO_3 2.25 M solutions, or below, execute the emptying process while 2.5 M solutions maintain conditions inside the ampulla unchanged. With NaNO_3 and NaCl 1.25 M solutions or below, evacuate the ampulla while 1.5 M solutions just keep conditions unchanged. With sucrose the corresponding concentrations are 1.75 M and 2 M respectively.

Moreover, the degree of evacuation was found to be inversely proportional to the molecular concentration of the solution. Text-fig. 4f shows the amount of sperm evacuated from a spermatophore kept in 1.5 M sugar solution over-

night. In such a solution complete evacuation was never obtained as an equilibrium between the two solutions on both sides of the inner layer was reached.

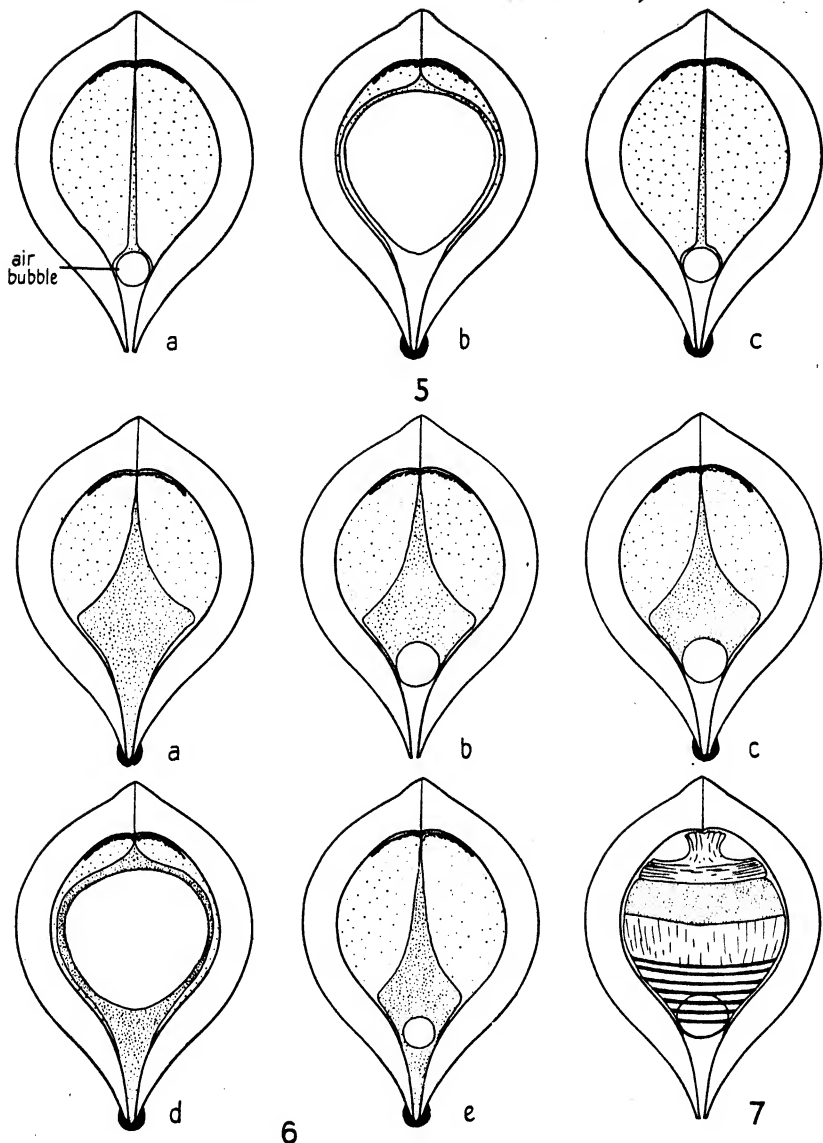
TABLE 1. *The Effect of Different Salt and Sugar Solutions on the Evacuation of Spermatophores*

Salt or Sugar	Molecular concentrations						
	1.0	1.25	1.5	1.75	2.0	2.25	2.5
MgSO ₄ . . .	+n	+	+	+	+s	+v.s	—
KCl . . .	+n	+	+	+	+s	+v.s	—
KNO ₃ . . .	+n	+	+	+	+s	+v.s	—
NaNO ₃ . . .	+s	+v.s	—
NaCl . . .	+s	+v.s	—
Sucrose . . .	+n	+	+s	+v.s	—

+, contents coming out; —, nothing comes out; n, flow is normal as compared with water; s, flow is slow; v.s, flow is very slow.

By using solutions of the same salts but of higher concentrations, and by introducing an air bubble the inner membrane can be forced to take its original position. A spermatophore was first deprived of the outer layer, taken out of the mould, had the tube cut somewhere between the handle and the ampulla and dipped in water; this produced quick and complete evacuation. When the ampulla became totally empty it was taken out, placed on a slide and left for a while. The ampulla eventually lost some of its water, allowing an air bubble to get between the two halves of the collapsed inner membrane (Text-fig. 5a). The open end was now sealed with paraffin wax, and the ampulla was dipped in any of the more highly concentrated solutions, e.g. 2.75 M of the first group or 1.75 M of the second group. The air bubble expanded and the inner membrane moved outwards and eventually regained approximately its original position (Text-fig. 5b). This took about 10 minutes. The material enclosed between it and the inner layer maintained, however, its transparent appearance. If the solution was replaced by water or any dilute solution the inner membrane was forced again to collapse and the air bubble to take its small original size (Text-fig. 5c). The whole process was repeated several times as the blocked empty ampulla was transferred from one solution to the other. Without an air bubble it was hardly possible to detect any movement of the inner membrane.

The experiment was repeated with an intact spermatophore evacuated by cutting the tube on a slide (Text-fig. 6). As has been mentioned above, complete emptying can never be obtained in this way. The remaining part of the granular substance was found to hinder the entrance of an air bubble. This difficulty was overcome by blocking the opening of the ampulla (Text-fig. 6a) as the flow stopped, and dipping it in a concentrated solution, e.g. 2.75 M of the first group. After a while the ampulla was taken out and the plug removed, when an air bubble immediately entered (Text-fig. 6b). The opening



TEXT-FIG. 5a. Represents a spermatophore ampulla that had had the outer layer removed, the evacuating fluid dried up and was emptied in water. An air bubble entered as the ampulla was left to dry. b, Shows the expansion of the air bubble when the ampulla was sealed and placed in a 2.75 M MgSO_4 solution. The inner membrane was forced to take approximately its original position. c, The air bubble resumed its original size as the ampulla was immersed again in water.

TEXT-FIG. 6a. Represents a spermatophore ampulla that has been evacuated on a slide by cutting the spermatophore tube somewhere between the ampulla and the handle. It was sealed and placed for a short time in a 2.75 M MgSO_4 solution. b, The seal was removed and an air bubble entered. c, It was sealed again. d, The air bubble expanded as the ampulla was placed in the 2.75 M MgSO_4 solution. e, The ampulla was returned to water. The inner membrane took nearly the position shown in a, and the air bubble was forced to take a smaller size.

TEXT-FIG. 7. Represents a spermatophore ampulla that had had the outer layer removed, the evacuating fluid dried up and placed in a 2.25 M sugar solution for 12 hours. The pressure body shrank and an air bubble entered as the ampulla was taken out.

was now sealed again and the ampulla placed in the solution. The same processes of expansion and contraction occurred when the ampulla was transferred from one solution to the other (Text-fig. 6c, d, and e).

A spermatophore that had had the outer layer removed and the fluid dried up could be preserved in any of the solutions which keep conditions inside the ampulla unchanged for a long time—a period of 2 weeks was tried—and still worked normally when water or any dilute solution was used. With regard to temperature, Regen found that spermatophores did not work when examined at low temperatures which he presumed would be near the freezing-point of the pressure body.

It is clear that the whole process is carried out by osmosis. The inner membrane moves inwards as water diffuses in and so exerts a pressure on the contents of the capsule, and moves outwards as water diffuses out. The inner layer functions, therefore, as a semi-permeable membrane separating two fluids of different osmotic pressure, the evacuating fluid and the pressure body. The difference between the osmotic pressure of the two fluids is very great. The depression of the freezing-point (Δ) was determined. Four specimens were taken of each and the result was as follows:

The evacuating fluid, $\Delta = 0.898^\circ \text{C}$

The pressure body, $\Delta = 3.062^\circ \text{C}$.

Water can diffuse through any part of the inner layer. This has been proved by covering its surface gradually with wax. Any uncovered area at any point on the surface, however small, will allow water to diffuse in or out. As a control ampullae that were covered totally with wax did not work.

MIGRATION OF SPERM TO THE SPERMATHECA

It remained to find out whether the sperm would migrate from the ampulla to the spermatheca if the spermatophore was deprived of the evacuating fluid. Sexually excited males were chosen and the outer layer of the spermatophore ampulla was removed and the fluid dried up. When the males had recovered from anaesthesia they were provided with virgin females. This was carried out in the hope that such treated spermatophores could be delivered to the females, but they were always pushed out and dropped by the formation of fresh ones. So the same operation was carried out immediately after the spermatophore was delivered. In order to prevent the females eating the spermatophores their heads were amputated and the cuts were sealed with wax. Four virgin females were treated in this way, and their spermathecae as well as the spermatophores were examined at intervals of $\frac{1}{2}$, 1, 6, and 12 hours after copulation. In every case almost the same amount of sperm was found in the spermatheca, but the delivery of the whole amount was never obtained. On the other hand it was found that in an hour the whole amount of sperm and most of the seminal fluid would migrate from an intact spermatophore to the spermatheca. So it is evident that in the four treated spermatophores certain amounts of sperm had migrated during the operation and

drying up of the evacuating fluid. When the ampulla became completely dry the flow of sperm almost certainly came to an end. Once the sperm reach the tip of the spermatophore tube they become very active, stimulated most probably by the spermathecal fluid, and carry on the remaining part of the way to the spermatheca apparently by their own movements. Sperm obtained from the spermatheca were more active in Ringer than those obtained from the spermatophore or the vesicula seminalis.

ACKNOWLEDGEMENTS

I am much indebted to Dr. W. H. Thorpe under whose supervision this work has been carried out, to Drs. M. G. M. Pryor and J. S. Kennedy for their valuable suggestions, to Dr. J. A. Ramsay for the freezing-point determinations, and to Mr. C. W. Coombs for reading the manuscript.

SUMMARY

The house cricket spermatophore is largely protein and does not contain any chitin. It is composed of an ampulla, a handle, and a long capillary tube terminating in a pointed closure.

The wall of the ampulla comprises four different layers: an outer thin layer, an evacuating fluid, a thick strong inner layer, and a transparent inner membrane. In between the inner layer and the inner membrane, at the top of the cavity, there is the pressure body, a protein mass, divided into two halves. Each half is surrounded by a thin membrane.

The process of emptying a spermatophore is due to osmosis. It has been shown experimentally that the strong inner layer of the ampulla acts as a semi-permeable membrane separating two fluids of different osmotic pressure—the evacuating fluid and the pressure body. When the closure at the tip of the tube is removed, the pressure body swells, exerting a pressure on the sperm as the evacuating fluid passes inwards.

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Further Remarks on the Golgi Element

BY

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INTRODUCTION

IN 1944 I published a long paper on the structure and chemical composition of the Golgi element. Since then the subject has been actively pursued in the Department of Zoology and Comparative Anatomy at Oxford by Dr. O. L. Thomas, Dr. A. J. Cain, Dr. Su-Hsuen Wu, and myself. In 1948 Dr. G. C. Hirsch visited us for discussion and practical collaboration. The main purpose of the present paper is to consider whether the view of the Golgi apparatus presented in the earlier paper is correct, and if not, how it should be changed. The opportunity is also taken to give the details of improved technique.

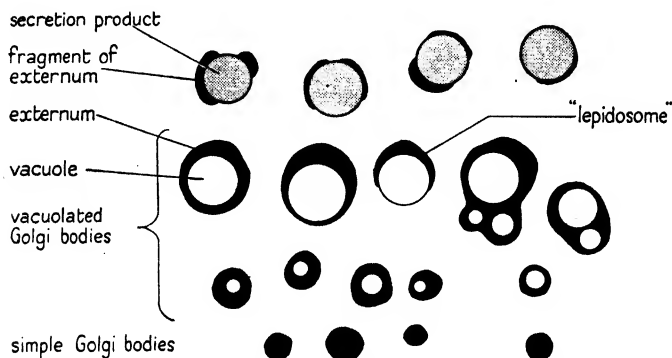
It is thought convenient to start by giving a general account of the Golgi element as it appears to me in the light of our researches, and by explaining the nomenclature that will be used. This will considerably simplify the exposition of the new results. The latter were obtained by a re-examination of the same kinds of cells as formed the basis of the 1944 paper. Some investigations of another kind of cell are mentioned on pp. 305-6.

THE STRUCTURAL PLAN AND NOMENCLATURE OF THE GOLGI ELEMENT

The name 'Golgi apparatus' applies strictly to a network of particles of silver seen in the cytoplasm of the Purkinje cells of the cerebellum of the barn-owl *Tyto alba* (called by Golgi (1898) *Strix flammea*), and of certain other nerve-cells, when particular reagents have been applied in a particular sequence. There is no evidence that any single constituent corresponding at all closely with this network exists in the living cell. Dr. Thomas's investigations (1948) suggest that it represents the Golgi bodies, in the [Quarterly Journal Microscopical Science, Vol. 90, part 3, September 1949]

restricted sense in which this expression will be used in the present paper, together with mitochondria and artifact (that is, particles of silver deposited at random on the surface of other particles already deposited). It is well known that the classical methods for the 'Golgi apparatus' often impregnate mitochondria. Ramón-Cajal himself freely allowed this, and illustrated mitochondria in the pancreas and submaxillary gland, beautifully impregnated by his uranofomol method (1933, Figs. 3 and 457).

This being so, one is tempted to discard altogether the name of Golgi when referring to the objects described in this paper. However, they must have a



TEXT-FIG. 1. Diagram illustrating the author's opinion as to the structural plan of the fully developed Golgi element, as seen in a very thin section.

distinguishing name, and it is true that in many cells the method of Golgi and the other classical methods do impregnate the region of the cell in which the Golgi bodies *sensu stricto* lie, more readily than they impregnate other cellular constituents (though they do not reveal the true structure of this region). It seems inevitable, therefore, that for the present, at any rate, the name of Golgi must be retained.

In what follows, the nomenclature adopted will be the simplest, most descriptive, and least tendentious that I can devise.

The word 'apparatus' has little to commend it. When one wishes to deal in the most general way with characteristic cellular constituents, it is legitimate to speak of nuclear, mitochondrial, and Golgi *elements*. The *Golgi element*, then, consists of separate *bodies*, which are rounded, but seldom perfectly spherical (see Text-fig. 1). There are usually many Golgi bodies in one cell. The Golgi body may be *simple*, i.e. apparently homogeneous, or it may be *vacuolated*, in which case it consists of an *externum* investing a *vacuole*. The lipid nature of the simple Golgi body and of the externum is revealed especially by its affinity for sudan black. When a series of stages in time can be observed, it is found that simple Golgi bodies occur first and vacuolated ones later. The latter almost certainly arise by vacuolation of simple bodies. The externum seldom covers the whole vacuole in a layer of even thickness:

the degree of its eccentricity in relation to the vacuole varies considerably in different cases, and sometimes it appears to be reduced to a small cap or band, though a very thin layer may perhaps even then extend over the whole vacuole. The vacuole is perfectly spherical; this fact, coupled with the impossibility of demonstrating any solidity in it, suggests its watery nature, which is the cause of its disappearance in routine microscopical preparations even when care is taken to preserve the lipid that surrounds it; the lipid is pulled about by protein precipitation in its vicinity and does not retain its shape exactly, and there is then nothing to suggest the previous existence of the vacuole. The vacuole has no affinity for sudan black.

One, two, or several vacuoles may be present in one body, which may thus be *uni*-, *bi*-, or *multivacuolate*.

In large vacuoles new substances, the *secretion-product*, may begin to appear. Very diverse substances appear in this way in the Golgi bodies of different cells, and no generalization can be made about them except that they are of protein or lipid nature. When the secretion product has been formed, the material of the lipid sheath does not form a complete covering. Eventually the secretion-product becomes free from any investment and no longer forms part of the Golgi element.

The simple Golgi body corresponds to the 'Praesubstanz' of my friend, Dr. G. C. Hirsch (1939), and the vacuolated body to his 'System'. My reasons for using a different terminology are these. The word Praesubstanz is not really applicable to a separate *object*, but only to the substance of which that object is made; and the word System is not purely descriptive, but involves theoretical considerations. The vacuole corresponds to the 'Inter-num' of several authors, but Dujardin's word (1835) is applicable here and is more descriptive.

The view of the Golgi apparatus just presented agrees in general with that put forward in my 1944 paper, but differs in the following points:

1. I no longer call the vacuole the 'neutral red vacuole'. It is true that it usually has a particular affinity for neutral red *intra vitam*, but as this is not invariably so, it seems better to discard the name.
2. I no longer place a diffuse lipid-containing substance between the vacuoles, because it cannot be seen in life and there are undoubtedly many cases in which it cannot be demonstrated by any means; and even when it seems to occur in fixed preparations, one may be deceived by the out-of-focus appearance of sudanophil material lying above or below. This is not a matter on which I would wish to dogmatize, but I no longer regard the diffuse lipid as a necessary constituent.

METHODS

Only the methods of general application are considered under this head. Special techniques, appropriate to particular tissues, are given in the descriptive part of the paper.

Vital Methods

In all the researches of my associates and myself, chief reliance has been placed on the study of the living cell. In this field the chief advance has been the use of phase-contrast microscopy. The method of Kempson, Thomas, and Baker (1948) has been adopted, with certain improvements that will be published shortly. Dark-contrast ('positive' phase-contrast) has on the whole been found preferable to bright in these studies.

Sodium chloride solution of the proper osmotic pressure, with the addition to each 100 c.c. of 0.2 c.c. of 10 per cent. calcium chloride solution, remains the standard medium for the examination of living cells. I have tried more complex solutions without noticing the least difference in the structure of the Golgi bodies, with the possible exception that Hédon-Fleig saline may be preferable for the cells of the snail.

Dahlia and neutral red (chloride) remain the most useful vital dyes. These are kept in solutions at 0.5 per cent. in distilled water. When required, 0.1 c.c. of the dye solution is added to 5 c.c. of the saline solution just described, and the cells are teased or otherwise immersed in this. Stronger and weaker solutions are used for particular purposes.

The Improved Sudan black Technique

Sudan black was introduced into biological technique by Lison and Dagnelie (1935). It was first used to show the Golgi bodies by myself (1944) and it has been adopted by my associates. Foster (1947) has used it in a study of the pituitary gland.

The design of the sudan black technique for the Golgi element rested on three principles: (1) that no protein precipitant should reach the cells until fixation was complete; (2) that the colouring agent should act simply by solution in the substance coloured; and (3) that the technique should present a picture of the Golgi bodies resembling that seen in living cells, so that it might be used with some confidence on cells that cannot be isolated for examination in the living state. I have now considerably improved the technique, while adhering to these principles. The new technique bears a resemblance to that of Ciaccio (1910).

The intention has been to reduce the tendency of sudan black to colour mitochondria and ground-cytoplasm, while increasing its tendency to colour the simple Golgi bodies and externa. The new technique depends upon the fact that treatment of the tissue in bulk with potassium dichromate greatly facilitates the subsequent colouring of these cellular constituents with sudan black, as I found some years ago (see Cain, 1947, p. 152), while formaldehyde antagonizes the subsequent colouring of the mitochondria and cytoplasm by sudan black. The technique is designed in such a way that the actions of potassium dichromate and formaldehyde are balanced against one another. In a successful preparation simple Golgi bodies and externa are dark blue or blue-black, while the vacuole is colourless and the cytoplasm either colourless or nearly so.

The following solutions, &c., are required:

Formaldehyde-saline

Formalin	10 c.c.
Sodium chloride, 10 per cent. aq.	7 c.c.
Distilled water	83 c.c.

Keep marble chips in the solution.

Dichromate-formaldehyde

Potassium dichromate, 2.5 per cent. aq.	88 c.c.
Sodium chloride, 10 per cent. aq.	7 c.c.

To 19 c.c. of this stock solution add 1 c.c. of neutral formalin. (Keep marble chips in the formalin bottle to maintain neutrality.)

Potassium dichromate, 5 per cent. aq.

Gelatine for embedding

Soak 25 gm. of powdered gelatine for an hour in 100 c.c. of a 0.2 per cent. aqueous solution of sodium *p*-hydroxybenzoate or Moldex. (The latter is obtainable from R. Campbell, 7 Idol Lane, London, E.C. 3.) Then warm in an incubator maintained at 37° C. till the gelatine has dissolved; filter through muslin while still warm. (The sodium *p*-hydroxybenzoate or Moldex prevents the growth of moulds and bacteria. It is superior to cresol in preventing the growth of the bacterium that liquefies gelatine gels.)

Formalum

Formalin	20 c.c.
Potassium alum, 5 per cent. aq.	80 c.c.

Keep marble chips in the solution. This new agent for hardening gelatine will be found useful by workers with the freezing microtome. Both gelatine blocks and sections may be preserved indefinitely in it. It facilitates the cutting of thin sections, as it makes the gelatine very hard; and the sections are not sticky. (Formalum must not be used in the acid haematein test for phospholipines (Baker, 1946), as the alum would react with the haematein.)

Sudan black

A saturated solution in 70 per cent. alcohol. It suffices to allow 0.5 gm. to 100 c.c. It is essential that the solution should be thoroughly saturated. If a solution is required quickly, boil for 10 minutes with reflux condenser.

The following specimens of this colouring agent are good:

- Sudan black 404194/480705 of the British Drug Houses;
- Sudan black No. 2 of E. Gurr;
- Sudan black 349 of W. T. Gurr;
- Sudanschwarz B N of Höchst.

Some of the specimens of sudan black on the market are useless for this technique.

Mayer's Carmalum (Mayer, 1892).

Farrants's medium (can be obtained ready-made from G. T. Gurr).

- I. Fix a piece of tissue not more than 3 mm. thick in formaldehyde-saline for 1 hour.
- II. Transfer without washing to dichromate-formaldehyde and leave for 5 hours.
- III. Transfer without washing to 5 per cent. aqueous potassium dichromate and leave overnight (about 18 hours) in this solution at room temperature.
- IV. Leaving the tissue in the same solution, transfer to the paraffin oven at 60° C. and let it remain there for 24 hours.
- V. Wash for 6 hours in running water.
- VI. Leave overnight in the prepared gelatine, melted at 37° C.
- VII. Cool the gelatine (preferably in a refrigerator), cut out a rectangular block containing the specimen, and place it in formalum. Place a marble chip in the capsule or tube. Leave overnight (or any convenient longer period).
- VIII. Cut sections at 8 or 10 μ on the freezing microtome.
- IX. Transfer a section to 70 per cent. alcohol. It is best to transfer sections from fluid to fluid, up to stage XV, in a porcelain pot with small holes in the bottom (e.g. the Royal Worcester porcelain thimble No. a.4756, Size 2, obtainable from Messrs. A. Gallenkamp).
- X. Transfer to the sudan black solution and leave for $\frac{1}{2}$ –4 minutes. (The best period is usually about 2 $\frac{1}{2}$ minutes.)
- XI. Wash in 70 per cent. alcohol for 5 seconds.
- XII. Wash in 50 per cent. alcohol for 1 minute.
- XIII. Wash in water. Sink the section if it floats.
- XIV. Transfer to Mayer's carmalum and leave for 2–4 minutes. (The best period is usually about 3 minutes.)
- XV. Rinse in distilled water. (A precipitate may form if tap-water is used at this stage.)
- XVI. Remove the section from the porcelain pot and wash for about 2 minutes (or any convenient longer period) in a tongue-jar or other fairly large bowl of water (tap or distilled: it makes no difference).
- XVII. Wash again in a large bowl of water.
- XVIII. Transfer to a Petri dish of water. Float the section on to a glass microscopical slide. Remove the slide from the water.
- XIX. Mount in *Farrants's medium*.
- XX. Attach a clip to hold the cover-glass to the slide. Leave overnight in the paraffin oven, to harden the mounting medium. (One may examine the slide after a quarter of an hour and then return it to the oven to complete the hardening.)

Result. If successful, the technique gives this result: simple Golgi bodies and externa, dark blue or blue-black; Golgi vacuoles, colourless; cytoplasm, colourless or pale grey-blue; chromatin, pink or red.

Never attempt to judge the colouring until the section is mounted. An immersion-objective must then be used. If the result is not good, try another section, using different periods in the colouring agents.

The only difficulty in this technique is caused by the fact that the sudan black solution must be saturated. As a result, the slightest evaporation causes a precipitate; this, if it occurs, will be found on the surfaces of the section, or occasionally, in loose tissues, between the cells: it never occurs within cells. To minimize this trouble, be careful to keep the sudan black solution in a stoppered jar while the section is being coloured.

If there is difficulty in colouring the Golgi bodies, it is worth while to repeat the whole process with the omission of stage I; that is, fix the tissue directly in dichromate-formaldehyde.

No claim is made that simple Golgi bodies and externa are in every case exhibited by the sudan black technique, nor that a dark blue or blue-black coloration necessarily indicates a Golgi body: for instance, triglycerides react in the same way. It is useful to fix a piece of tissue in formaldehyde-saline and colour a section with sudan IV; this will show triglycerides and certain other lipoids, but not Golgi bodies. When triglycerides are very abundant in a cell, they interfere with the technique.

All that is claimed for the method is that when it is used on kinds of cells that can also be examined alive, it gives pictures that resemble the living condition fairly closely; that it often shows Golgi bodies and externa very well; and that it is not 'capricious' nor 'patchy' in its effects, as the classical methods for the Golgi element are, for it can be relied on to give the same appearance every time.

I hope that anyone who decides to try this technique will learn it upon an 'easy' tissue. I particularly recommend the intestine of the mouse: it is scarcely possible to fail with this. Cut out a piece of empty intestine about a centimetre long and drop it into the formaldehyde-saline. After 5 minutes (when the outer layers have hardened and the tissue is less likely to lose its form), open it by a longitudinal cut from one end to the other, taking great care not to do any unnecessary damage to the villi. Leave the section only 1 minute in sudan black and 2½ minutes in carmalum. The resulting preparation should show the Golgi bodies clearly in every cell of the epithelium and of the glands of Lieberkühn.

Those who try this technique may like to try also that of Thomas (1948), who colours the Golgi bodies with sudan black in paraffin sections.

RESULTS

The Epithelial Cell of the Intestine of the Mouse

It is much easier to display the epithelial cell of the intestine of a mammal for *intra vitam* study, than the corresponding cell of the newt; and I have therefore changed to the study of the former.

To display this cell in the unfed mouse, proceed as follows: Choose a part of the intestine that is free from food and cut out a piece about a centimetre

long. Place it on a glass plate and open it by a longitudinal cut with scissors; lay it flat, with the inner side uppermost. Keep it moist with a few drops of the saline solution (p. 296). Holding one end of it still, scrape it several times with a sharp straight scalpel; the villi will be cut off and will accumulate near the edge of the scalpel. Wash them with saline on to a microscopical glass slide; cover; ring the coverslip with vaseline.

The villi will be seen under the low power of the microscope as objects resembling in shape the last joint of a finger. Place the middle of a villus in the optical axis and adjust for phase-contrast microscopy with the oil-immersion $\frac{1}{2}$ in. or $\frac{1}{4}$ in. objective, using dark contrast ('positive' phase-contrast).

The free border of the cell lies towards you. Focusing up from near the middle of the villus, you will see first the basal group of mitochondria, then the nucleus, then the Golgi bodies, then the apical group of mitochondria (these are the hardest to see), and finally the intercellular band ('Kittelleiste') and free border. *The Golgi bodies are the most evident objects in the cell.* They are situated where the classical methods show a network, but have quite a different structure.

The bodies are of various sizes, the largest being about 2μ in diameter. The largest contain more than one vacuole, while the small ones have a single vacuole or none. The material of the simple Golgi bodies and of the lipid sheaths appear dark by 'positive' phase-contrast microscopy. It is not necessary to show a figure of these bodies, for they are sufficiently well represented by the univacuolate body on the left of Text-fig. 1 and the bi- and multi-vacuolate ones on the right.

Nothing is seen resembling a fully formed secretion-product.

The same appearance is seen if the villi, after being cut off, are soaked for 15 minutes in one of the vital dyes (p. 296) in a watch-glass, with occasional stirring, and then examined by ordinary microscopy. Both dahlia and neutral red colour the Golgi bodies. The exact distribution of the dyes is difficult to determine, but the dahlia certainly stains the externum blue. In the vacuolated bodies neutral red colours the vacuole, but I cannot state with confidence that it does not also stain the externum.

It may be recalled that in the newt, dahlia stains the externum blue and the vacuole yellow or red; neutral red colours the vacuole only (Baker, 1944).

The sudan black technique shows the Golgi bodies of the intestinal epithelial cell of the mouse very much as they appear in life. The sudan black colours only the lipid material, leaving the vacuoles colourless. The only obvious difference from the living state is that the bodies are nearer together (presumably on account of the contraction of the cytoplasm). The vacuoles are sometimes rather difficult to see.

The Primary Spermatocyte of the Common Snail, Helix aspersa.

In the very young primary spermatocyte, in which there is as yet very little cytoplasm, there are only a few small simple Golgi bodies, uniformly coloured by sudan black.

It is necessary to correct in several respects the account of the Golgi bodies in the large primary spermatocyte given in my 1944 paper. Dr. Wu and I find that neutral red, especially when used at higher concentrations than that suggested on p. 296, gives rise to pink or red vacuoles where none existed before. These vacuoles usually arise in the Golgi region, sometimes in contact with the 'lepidosomes'; but they sometimes arise in the Golgi region not in contact with any lepidosome, and, when high concentrations of the dye are used, they may be seen to form in other parts of the cytoplasm and to grow gradually to a considerable size. It is therefore clear that judgments as to the structure of Golgi bodies should not be made until a cell has been carefully studied in life, without the use of neutral red. It must, however, be mentioned that the production of artificial vacuoles by neutral red does not occur in most cells: the spermatocyte of the snail is exceptional in this respect.

At first sight there appears to be nothing exactly corresponding to the typical Golgi body in a large primary spermatocyte. Instead one sees the 'banana-shaped' lepidosomes, dictyosomes, or batonnettes. The name *lépidosomes* was given by Parat (1926) from their resemblance to *écailles*, and he and Painlevé (1926) remark that these bodies are often *squameux*; but he nowhere gives a careful description or figure that really substantiates these words. The lepidosomes, which are easily seen without staining, are readily coloured by dahlia and many other basic dyes *intra vitam* (never by neutral red), and by sudan black in fixed preparations. When they are attentively studied, one notices that it is very difficult to form a true opinion of their shape. As one focuses the microscope up and down with a delicate fine adjustment, the image of the lepidosome often moves a considerable distance, though the cell remains perfectly still. The banana-shaped appearance is a view in optical section and does not properly represent the whole object, which must be to some extent spread out like a scale. At my request, Dr. A. J. Cain has carefully studied the apparent movement of the lepidosome when one focuses up and down, and he confirms that the appearance can only be explained by the hypothesis that the object is commonly scale-like, and not shaped like a banana or rod. Indeed, Parat's name is a good one, though he did not himself fully explain his reasons for choosing it.

In an unstained living cell the lepidosomes appear grey. It seems certain that this is because they are of higher refractive index than the surrounding medium, and so shaped as to refract some of the light coming from the condenser of the microscope in such a way that it does not enter the objective. A bright line is usually seen on the concave side of the lepidosome. It is not necessary to suppose that this represents another constituent of the object, though Dr. Wu was inclined to think so; rather it represents a region of the lepidosome that acts to some extent as a lens in converging light into the objective. Such alternations of dark and light must occur whenever an object with curved outlines is made of a material of higher refractive index than its surroundings. The bright lines move with the dark when the focus

of the microscope is changed. The fact that the lepidosome has a high refractive index is shown by its appearing dark when examined by 'positive' phase-contrast; further, its affinity for sudan black shows it to contain or consist of lipid. The bright line is not seen when phase-contrast is used.

If one examines a medium-sized lepidosome with the utmost care, focusing continually up and down, one reaches the conclusion that it is *a mass of matter applied to parts of the surface of an invisible sphere*: I can think of no other description that is adequate. When phase-contrast is used, the sphere can sometimes (but by no means always) be seen. I have shown it to Mr. P. C. J. Brunet and Dr. A. J. Cain, who allow me to say that they have seen it with perfect clarity; they have both drawn the object as a sphere with a dark mass spread over part of the surface. Our studies strongly suggest that this structure exists, or is readily assumed, during the latter part of the meiotic prophase. It was, indeed, seen long ago by Perroncito (1910) in *Vivipara vivipara*, especially when the primary spermatocyte was actually dividing (see his Fig. 31 on Plate 1); but he made the mistake of supposing that he was seeing rings or disks.

Exceptionally large lepidosomes seem not to fall within these generalizations: parts of them may perhaps hang free, not applied to the surface of a vacuole. They are too infrequent, however, to be subjected to detailed study by different methods.

The sudan black technique was used for the study of the primary spermatocyte in sections. Great care must be taken in dissecting out the ovotestis; otherwise some of the nurse-cells will be damaged and their lipid droplets, coloured by sudan black, will lie about in the alveoli and tend to obscure the spermatocytes. The period of colouring in sudan black should be reduced to $1\frac{1}{2}$ minute.

In sections coloured with sudan black the Golgi body is not so difficult to interpret as in the living state, for the uncoloured vacuoles are clearly seen, with their lipid sheaths. It would appear that fixation has somehow spread the lepidosome farther over the surface of the vacuole, and thus made the whole appearance much more similar to that seen in life in other kinds of cells; the very sharp distinction between the material of the lepidosome and the white of the vacuole helps to make everything clear.

If this interpretation is correct, the lepidosome is an externum of a special kind: it is spread on rather a small part of the surface of a vacuole that is difficult to see in life and does not stain with neutral red. (See the diagram of a lepidosome in Text-fig. 1.)

It is to be observed that this view of the Golgi apparatus of the primary spermatocyte of the snail accords fairly closely with that of Monné (1938, 1939). The main difference is that he considered the vacuole to be of the shape of a biconvex lens, not a sphere. He regarded the lepidosome as spread on its surface, being particularly thick round the rim of the lens; and he showed that when the living cell is treated with ammonium chloride solution, the material constituting the lepidosome distributes itself nearly evenly over

the vacuole, so that an appearance resembling the familiar type of Golgi-body is produced.

In the shortlived secondary spermatocyte the form of the Golgi bodies resembles that seen in the primary. In the spermatid the Golgi bodies clump together at first and then often separate while taking up their position well behind the nucleus; the very small acrosome meanwhile appears at the front end of the spermatid, without any apparent connexion with the Golgi bodies. We are not able to offer any suggestion as to the function of the Golgi bodies in this particular spermatogenesis. It is well known that in very many cases they migrate to behind the nucleus after secreting the acrosome, and some of them remain there while others are thrown off with the residual cytoplasm. The evidence from *Helix* suggests that the Golgi bodies may play a part in the development of the region of the spermatozoon posterior to the nucleus; for it is not likely that such a large cellular constituent should be functionless. This suggests that in the spermatogenesis of most animals the Golgi bodies may have another role besides that of secreting the acrosome.

Dr. Wu and I devoted a considerable amount of time to an attempt to learn something of the structure of the Golgi bodies in primary spermatocytes by varying the pH and osmotic pressure of our saline solutions. Unfortunately we were not able to obtain any information in this way. We found the Golgi bodies remarkably resistant, maintaining their form even when the cytoplasm was greatly shrunken or swollen. Monné (1938) had already demonstrated their resistance to hypertonic solutions in the case of this particular cell.

The Nerve-cell

In my 1944 paper I put forward the opinion that in the cells of the anterior mesenteric ganglion of the rabbit the Golgi bodies are represented by sub-spherical objects, each consisting of one or more vacuoles surrounded by a sudanophil externum. This opinion was in sharp opposition to the generally accepted opinion as to the structure of the Golgi element in nerve-cells. It was generally supposed that the true structure was a network.

Dr. O. L. Thomas devoted his two years of study at Oxford to the cytoplasm of nerve-cells. I followed in detail every stage in his work; but as I have not pursued the investigation of the nerve-cell in my own researches, this section of the paper will be short. The reader is referred to Dr. Thomas's papers (1947, 1948). He worked with both living and fixed cells. In his study of the nerve-cell of the anterior mesenteric ganglion of the mouse he confirmed my findings on the rabbit in every respect, except that he saw no special region of diffuse lipid in the cell; and, as has been remarked above, I no longer regard this as a necessary constituent of the Golgi element. In his general study of the cytoplasm of nerve-cells, however, he went very much farther than I did. He showed how a secretion-product appears within the vacuole, as though the cell were a gland-cell. He also made a careful study of the gradual deposition of osmium hydroxide in the cell in the Mann-Kopsch technique. He showed how the externa are blackened first, then the

interiors of the Golgi bodies, and then the mitochondria; finally a non-specific deposition occurs, which thickens the already blackened objects and joins them together into the well-known net.

Cain (1948) confirmed Thomas's work, so far as it referred to neurones of *Helix*, and extended it to other genera of pulmonate gastropods. He found that the orange substance ('lipochrome' of previous authors), which accumulates in the vacuoles of the Golgi bodies of these animals, is carotenoid.

Remarks on Chemical Composition

In my 1944 paper I brought forward strong evidence from various sources, not amounting to histochemical proof, that the lepidosomes of the spermatoocytes and spermatids of the snail consist of or contain lipine. In a review of the subject I showed that up to that time no real evidence had been produced as to their chemical composition, beyond the fact that they consisted of or contained a lipid, and even this knowledge rested not on secure histochemical foundations, but mainly on the observation that they are not usually seen after routine histological treatment involving the use of lipid-solvents. The use of sudan black demonstrates the lipid nature of simple Golgi bodies and externa. Dr. A. J. Cain (1947), by employing the acid haematein test (Baker, 1946, 1947), was the first to obtain further information by means of strict histochemistry. He demonstrated the presence of phospholipine in the Golgi lipid of the epithelium of the alimentary canal of the leech *Glossiphonia*. Phospholipine has been detected in the same way in the Golgi bodies of the nerve-cell of *Helix* by Thomas (1947) and Cain (1948), and in sebaceous cells of man by Montagna *et al.* (1948).

It is noteworthy that in the majority of cases the acid haematein test gives a negative result with Golgi bodies, while mitochondria react positively. It seems certain that there is some other lipid constituent beyond phospholipine, and it is a matter of great importance to discover what this constituent is. We only cover up our ignorance by speaking of 'Golgi material'. What is wanted is knowledge expressed in terms that mean something to a chemist.

DISCUSSION

There is no cellular constituent that is regarded with such a widespread feeling of uncertainty as the Golgi element. The reason for this is evident enough. The classical methods used in its investigation are no longer of much value except for directing attention to a particular part of a cell: they are neither morphological nor histochemical. Scarcely any attempt has been made to show that they reveal, in its natural form, something already existing in the cell during life. Instead, there has been a tendency to work towards a desired end: if the final preparation shows a desired picture, it is 'good'. The methods give no chemical information except the fact that, under certain arbitrarily chosen conditions, silver nitrate or osmium tetroxide is reduced. This, in itself, does not permit any valid deduction to be drawn as to the chemical composition of the objects blackened. Work of this calibre is not

recognized in any other branch of science, and it is natural that there has been a strong reaction against it, especially in America. There has been a feeling that the subject is unworthy of serious consideration.

Parat (1928) was the first to undertake a comprehensive scientific investigation of the subject, by directing his microscope towards that part of the living cell in which the classical methods reveal the 'apparatus'. His great contribution was the finding that vacuoles can generally be discovered in this region. He called them the neutral red vacuoles because they usually stain readily with this dye *intra vitam*. Accoyer (1924) had already suggested, on insufficient evidence, that there are two characteristic inclusions in the cytoplasm of animal cells: mitochondria, and vacuoles staining with neutral red.

It has been the policy of my associates and myself to follow the lead of Parat by basing our evidence as to structure mainly on observations on the living cell. We differ from Parat in laying stress on the lipid material inside which the vacuole appears, and which afterwards forms an externum around it.

The Golgi element is no longer inaccessible to investigation by strictly scientific methods, and it is legitimate to suggest that the reaction against its study has now gone too far. Many cytologists are delving into the sub-microscopic morphology of the cell, while a relatively enormous object stares them in the face, unnoticed. There is no intention here to try to detract from the value of submicroscopic investigation, but too many workers are prejudiced against devoting themselves to the Golgi element by reasons that are no longer valid.

A great deal of the mystery surrounding the Golgi element disappears if we look at it in the following way. When a secretion droplet is formed inside a cell, it does not usually appear loose in the cytoplasm. On the contrary, a vacuole first appears within a lipid particle, and this vacuole grows until the lipid is seen as a sheath round it; the secretion-product accumulates within the vacuole, and the sheath is at last thrown off.

Three main problems await solution:

1. What is the chemical constitution of the substance that forms the simple Golgi body and the externum round the vacuole? (See p. 304.)
2. What is synthesized within the vacuole in cells that make no obvious secretory product?
3. Does the simple Golgi body ever arise *de novo* in the cytoplasm? If not, do simple Golgi bodies always arise from pre-existing simple Golgi bodies? or do they arise from fragments of externa dropped off from fully formed secretion-product?

With regard to the last question, I have refrained from making Text-fig. 1 hypothetical by bringing an arrow round from the sheath fragments adhering to the secretion-product to the simple Golgi bodies, and thus completing a cycle. I have made some observations that bear upon this problem. In an unfed mouse, the amount of sudanophil material in the Golgi element of the pancreas is very small. Inject the animal subcutaneously with pilocarpine,

and an hour and a half later there is a great quantity of sudanophil material in the form of Golgi bodies, many of which are large and multivacuolate and resemble the figure given by Worley (1944) of a Golgi body in a living pancreas-cell of the frog tadpole, stained by methylene blue. It is clear that there has been a great synthesis of lipoid, preparatory to the synthesis of zymogen. A few hours later the cell is stuffed with zymogen droplets, with only a fragment of sudanophil material still attached to their surfaces here and there. It is clear that a rapid lipoid-metabolism is in progress. It is very difficult to design a method of following the details of that metabolism, but whoever succeeds in doing it in any cell will have solved the main problem of the Golgi element.

ACKNOWLEDGEMENTS

My colleagues, to whom I owe a lot, are mentioned in the first paragraph and elsewhere in this paper.

Dr. Su-Hsuen Wu came to Oxford to make a thorough reinvestigation of the Golgi element in the primary spermatocyte of the snail, in collaboration with myself. Almost the whole of our joint work was done on living cells. It was intended that we should both continue the work by studying fixed material after her return to China; but she must have reached Peking just before the city was captured, and I have not been able to get in touch with her. I earnestly hope that I may be able to collaborate with her again one day. Meanwhile I can only pay tribute to the remarkable perseverance and independence she showed in her determination to arrive at the truth about the nature of the object investigated. Under the circumstances I believe that she would wish me to publish our results. Whatever there may be of value in the account of the living primary spermatocyte of the snail is due more to her than to me.

We have received all possible help and encouragement from Professor A. C. Hardy, F.R.S. I must particularly mention Dr. G. C. Hirsch. His book (1939) has long been a source of inspiration; collaboration and discussion with him have been of immense benefit. I thank also Miss O. Wilkinson and Miss M. Le Bechee for much skilful practical assistance, willingly given.

SUMMARY

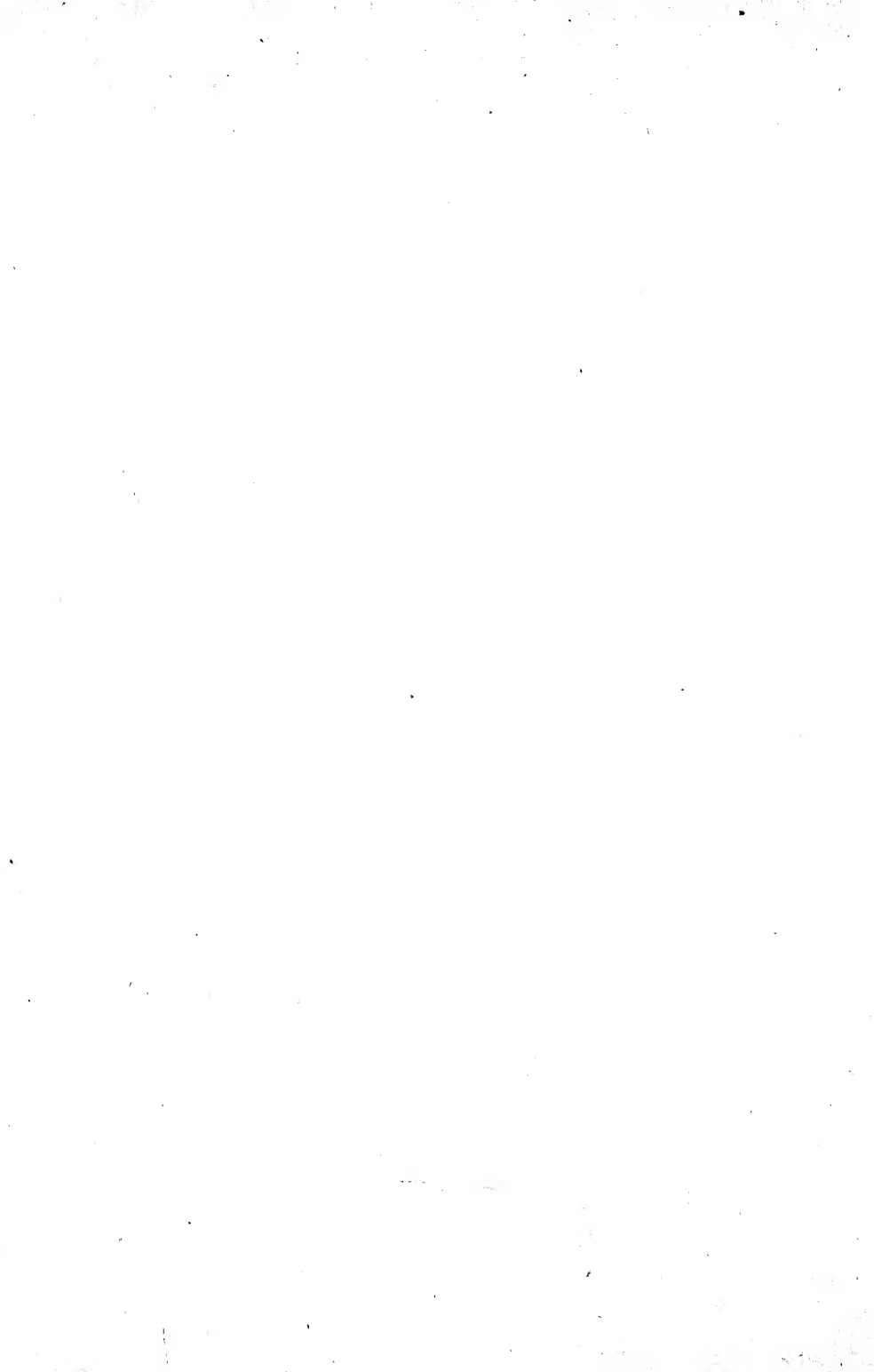
1. The Golgi element has been reinvestigated in the same kinds of cells as were the subject of the author's 1944 paper.
2. Two new methods have been used, namely, phase-contrast microscopy and an improved form of the sudan black technique, in which the tissues are postchromed at 60° C.
3. The Golgi element consists of separate bodies, spheroid in shape. These Golgi bodies may be simple (i.e. non-vacuolate), or may contain one or more vacuoles. The material of the simple Golgi body and of the externum of the vacuolate body is a lipoid that in some cases can be shown to contain lipine. The secretion-product of the Golgi body originates in the vacuole.

4. The opinion as to the structural plan of the Golgi element set out in the earlier paper has been confirmed in the main. There are, however, two exceptions to this:

- (a) The vacuole in the Golgi body does not invariably colour with neutral red, and this dye occasionally causes the appearance of vacuoles not present before, both within the Golgi region and in other parts of the cytoplasm.
- (b) 'Diffuse lipoid' is not a characteristic feature of the Golgi element.

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Aldehydes in relation to Absorption of Fat from the Intestine and Metabolism of Fat in the Liver

BY

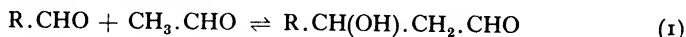
J. F. DANIELLI

(From the Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, London, S.W. 3)

With one Plate

INTRODUCTION

THAT aldehydes may be concerned in the synthesis and degradation of fat has often been speculated upon in the past. For example, the intervention of the aldol condensation



is compatible with most of the known facts about fat metabolism. In particular, it offers a ready explanation of the fact that the natural fatty acids have, almost invariably, an even number of carbon atoms, and of the fact that degradation of these acids normally occurs by loss of two carbon atoms at a time. But attempts to discover acetaldehyde in tissues, in relation to fat metabolism, have not been successful, and there are very few observations in the literature on the occurrence of longer-chain aldehydes. However, recent studies have shown that acetyl phosphate, $CH_3.CO.OPO_3H_2$, is a common constituent of tissues. The absence of resonance in this molecule (compared with that possible in free acetic acid) suggests the possibility that acetyl phosphate, or a related radicle, could replace acetaldehyde in equation (1). This being so, it becomes of great importance to investigate the occurrence of long-chain aldehydes in tissues, in relation to fat metabolism. In this paper attention is focused on the liver of the rat and the mouse. In this organ there is a close cytological association between sites of fat metabolism and sites of long-chain aldehydes. Failure to appreciate this in the past was probably caused by the ease with which oxidation of aldehyde to the corresponding fatty acid occurs during the usual procedures of the biochemist. By the use of cytochemical methods this difficulty is largely obviated.

In addition, preliminary experiments suggested that there is a relationship between aldehyde formation and the absorption of fat by the intestinal mucosa. Studies were therefore made of this point.

CYTOCHEMICAL METHODS

Tissues were studied for the distribution of alkaline phosphatase by the method of Takamatsu (1939) and Gomori (1939), as revised by Danielli (1946), and for the distribution of long-chain aldehydes by the method of Feulgen and Voit (1924) as revised by Danielli (1949). For demonstration of

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phosphatase paraffin sections were cut at 5μ , and for demonstration of aldehyde frozen sections were cut at 10μ and 20μ with liver, and at 15μ with intestine.

Fat was stained in the frozen sections with Scarlet R (Gurr), using the stain in 70 per cent. alcohol for 1 hour at room temperature, and differentiating in 70 per cent. alcohol. This method can be used successfully after carrying out the cytochemical test for aldehydes. Counterstaining with toluidine blue was found useful.

TREATMENT OF ANIMALS

The animals used were rats and mice. When placed on a special diet they were weighed daily. The normal diet contained carbohydrate, fat, and protein. The special carbohydrate diet contained 76 per cent. sucrose, 15 per cent. agar, 5 per cent. yeast, 4 per cent. salt mixture: animals on this diet were given a supplement of vitamins A, D, and E daily. All animals, including those starved, were allowed free access to water.

THE ABSORPTION OF FAT FROM THE INTESTINE

When animals were kept on a normal diet, which contained rather a small amount of fat, only occasional fat droplets were found in the intestinal mucosa. The intracellular droplets were covered with a spherical shell of aldehyde, as also were those droplets observed in the lacteals. In addition there was frequently a diffuse distribution of aldehyde between the nucleus and basement membrane of the cells making up the intestinal epithelium. When animals were kept on the carbohydrate diet, the intestinal mucosa was comparatively lacking in aldehyde and, as would be expected, no fat was found in the epithelial cells or in the lacteals. These preliminary observations suggest that aldehydes might be involved in the absorption of fat by the intestinal mucosa.

To test this hypothesis, animals which had been starved overnight were given by stomach tube a dose of 1 ml. of an emulsion of olive oil in water. The emulsion contained 50 per cent. of olive oil, with sufficient sodium bicarbonate to give a relatively stable emulsion. These animals were killed at intervals of 1, 2, 4, and 6 hours after administration of fat. When frozen sections from the intestines of these animals were examined, the intestinal mucosa cells showed a very heavy absorption of fat, especially in the case of those animals which were killed after 4 hours and 6 hours. It was commonly found that different regions of the epithelium would show quite different stages in the absorption of fat. In some cases the cells and lacteals were free from fat except for a few droplets just inside the free border of the cells; in some cells droplets were to be observed throughout the cell, in others the greatest concentration of droplets was proximal to the basement membrane; in some regions of the intestine fat droplets would be observed in the mesenteries only. When these sections were stained, aldehyde was found only in animals which were killed a short time after administration of fat. It thus was clear that the absorption of triolein does not occur by any stage involving the intervention of aldehydes. The fact that aldehyde was to be observed only

in the animals which were killed soon after dosing suggests that the aldehyde is formed in the mucosa cells in connexion with processes other than the absorption of fat, that when fat enters the cell the long-chain aldehydes are adsorbed upon the surface of the fat droplets, and are carried out of the cell by the fat droplets as these droplets pass into the lacteals; i.e. the absence of aldehyde in the intestinal mucosa cells towards the end of the experiment is probably attributable to a process of washing-out of aldehyde by the passage of oil droplets through the cell.

Whilst it seemed clear that aldehyde is not involved in the absorption of triglyceride, there still remained the possibility that it might be concerned with the absorption of free fatty acid. To test this animals were given 1 ml. of a suspension of sodium oleate in water; the suspension contained 50 per cent. of oleic acid by volume, adjusted to pH 6 by addition of sodium hydroxide. These animals were killed 1, 2, 3, 3½, 4, 4½, 5, and 5½ hours after administration of the fatty acid. The results obtained did not differ substantially from those found with triolein.

The conclusion thus appeared that long-chain aldehydes are not involved either in the absorption of fat from the intestinal lumen, nor in the passage of fat through the intestinal cells.

THE METABOLISM OF FAT IN THE LIVER

When the livers of animals on a normal diet were examined for the occurrence of aldehyde and of fat, it was found that the aldehyde was distributed around the intra-cellular fat droplets as a spherical shell. In addition there would often be a diffuse distribution of aldehyde in the cytoplasm, and sometimes this diffuse aldehyde was present in cells which appeared to contain no fat droplets. Considerable areas of the liver are often quite free from aldehyde. It was not clear, from these observations on animals on normal diet, whether the occurrence of the aldehyde was to be associated with the synthesis of fat or with the degradation of fat. Experiments were therefore carried out with animals fed on a diet which was rich in fat, on animals fed on a diet containing no fat but high carbohydrate, and on animals which were starved.

When the diet contained a considerable amount of fat, all the droplets found in the liver were surrounded with a spherical shell of aldehyde. This was also true of animals which had been starved for periods of up to 6 days. In the case of the starved animals it is known that the fat droplets appearing in the liver are derived from the fat depots, from which they pass to the liver. In the liver the fat is broken down into carbohydrate. Thus from these experiments it was clear that aldehyde is closely associated with the breakdown of fat to carbohydrate.

When animals are kept on a carbohydrate diet of the type used here, containing no fat, it is known that in so far as fat appears in the liver it is formed by synthesis of fat from carbohydrate. Experiments with tagged fat have shown that no fat passes from the fat depots to the liver, but fat may be synthesized in considerable amounts in the liver from carbohydrate. In the

experiments reported here, the usual response to placing an animal on carbohydrate-rich diet was a great decline in the quantity of aldehyde found in the liver. This was commonly associated with a parallel decline in the amount of fat to be observed in droplet form in the liver cells. After 2 or 3 days on this diet, aldehyde may begin to appear again in the liver. The greater part of this aldehyde is distributed diffusely in the cells, and not always in those hepatic cells containing fat droplets. The reappearance of aldehyde, however, was closely associated with the reappearance of droplet fat in the liver. The fat droplets frequently had spherical shells of aldehyde, but on the other hand it was quite common to find droplets which had no such shell of aldehyde. In some animals there was evidence of the secretion of aldehyde into the bile canaliculae.

It seems clear from these experiments that aldehyde is intimately concerned in the breakdown of fat, and may also be concerned in the synthesis of fat from carbohydrate, although the evidence for the latter conclusion is not so satisfactory as for the former.

Studies were made of the cytochemical distribution of alkaline phosphatase during fat synthesis and degradation in the liver. No evidence was obtained which would suggest that this enzyme is concerned in fat metabolism.

In some instances it was found that the bile canaliculae contained a concentration of aldehyde. This aldehyde is presumably secreted into the bile canals by the hepatic cells. The function of this activity is at present obscure.

In all experiments the fatty nature of the aldehyde was tested by extraction of the tissue sections with acetone before the performance of the cytochemical reaction. In all cases the aldehyde was extracted by acetone, showing that it is a fatty compound.

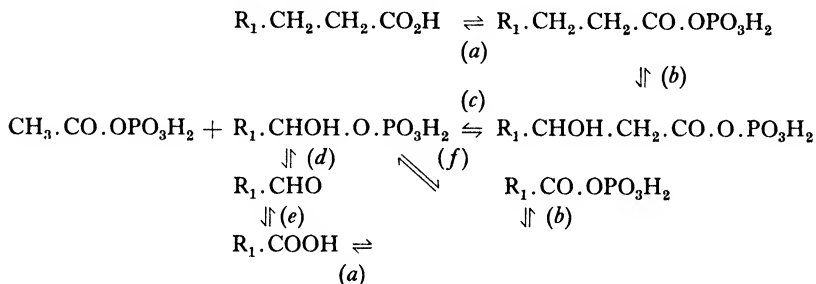
DISCUSSION

The results obtained in this work show that whilst long-chain aldehydes are probably not concerned in the absorption of fat from the intestine, they are intimately concerned with fat metabolism in the liver. Studies of a variety of tissues grown in tissue culture have shown that when fat is being laid down in cells, it is always surrounded by a spherical shell of aldehyde. These results will be published separately. The main conclusion to be drawn is that aldehydes are likely to be an essential intermediate in fat metabolism.

At present the details of the processes involving the synthesis and degradation of the higher fatty acids are obscure. It is known from extensive classical studies that a process of β oxidation is commonly involved, so that a series of 2-carbon fragments is lost from the fatty acid chain, rather than carbon dioxide. It is also known from recent work, particularly that of Lehninger (1945), that whereas free fatty acids are not readily attacked by the enzyme systems which can be extracted from hepatic cells, the acylphosphates are readily oxidized and are probably a main intermediate. To these conclusions must now be added the probability that the long-chain aldehydes are also a main intermediate.

As was pointed out in the introduction, it may be that not free aldehydes but phosphorylated derivatives of aldehydes are the true intermediates. If this is so the aldehyde found in the experiments reported here may be a breakdown product of an aldehyde phosphate. Aldehyde phosphates such as $R \cdot \text{CHOH} \cdot \text{OPO}_3\text{H}_2$ would certainly be unstable. Therefore two possibilities must be considered: (i) that free long-chain aldehydes are true intermediates; (ii) that free long-chain aldehydes are by-products or end-products of a synthetic cycle.

In case (i), in the degradation of a long-chain fatty acid it is possible that aldehyde phosphates and free aldehydes are involved in a series of reactions such as $(a) \rightarrow (b) \rightarrow (c) \rightarrow (d) \rightarrow (e)$ below

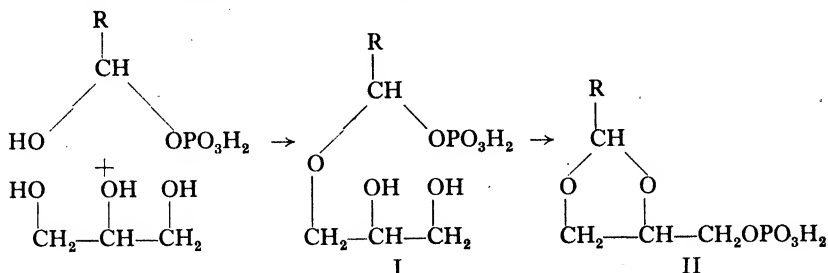


Alternatively, in case (ii) instead of the occurrence of reaction (d), which involves liberation of free aldehyde, aldehyde phosphate may be directly oxidized to fatty acid phosphate as indicated by (f). In the latter case free aldehyde would probably only appear as the termination of a series of synthetic reactions. A technique for the cytochemical demonstration of aldehyde oxidase would yield results which would assist in considering the likely sequence of intracellular reactions. At present no such technique is available. But it is known that there is a very high concentration of aldehyde oxidase adsorbed on the surface of freshly secreted milk fat droplets (Worden, 1943). It seems very unlikely that this is a fortuitous association. Furthermore, Prof. A. Worden informs me that there is also phosphatase adsorbed on the surface of milk fat droplets, which suggests a dephosphorylation is involved in at least one stage in fat synthesis. On the other hand, cytochemical studies made in the course of this work have failed to show any significant correlation between the occurrence of alkaline phosphatase and of fat metabolism.

A point which may prove to be significant was that each fat droplet undergoing degradation in the liver is surrounded by a spherical shell of basiphilic material, the site of which roughly coincides with the shell of aldehyde. The basiphilic character may be caused by nucleic acids, phospholipines, &c.

In conclusion it should be pointed out that, when it is realized that long-chain aldehydes or their derivatives are essential intermediates in fat metabolism, a new significance attaches to the occurrence of the aldehyde acetals

which were discovered by Feulgen and Bersin (1939) and which were discussed in a previous paper (Danielli, 1949). These acetals will arise, for example, by condensation of glycerol with aldehyde phosphate:



The condensation leading to substance I may well go spontaneously, since aldehyde phosphates would be highly reactive, and the wandering of the phosphate group to give II (the substance isolated by Feulgen *et al.*) may also be spontaneous, since it closely resembles the wandering of the phosphate group in the reaction α glycerophosphate \rightleftharpoons β glycerophosphate. It is reasonable to suppose that these acetals are intermediates in the synthesis of triglycerides and the phospholipins, &c., although the possibility must be borne in mind that the acetals may be also essential intermediates in the main channel of fatty acid metabolism.

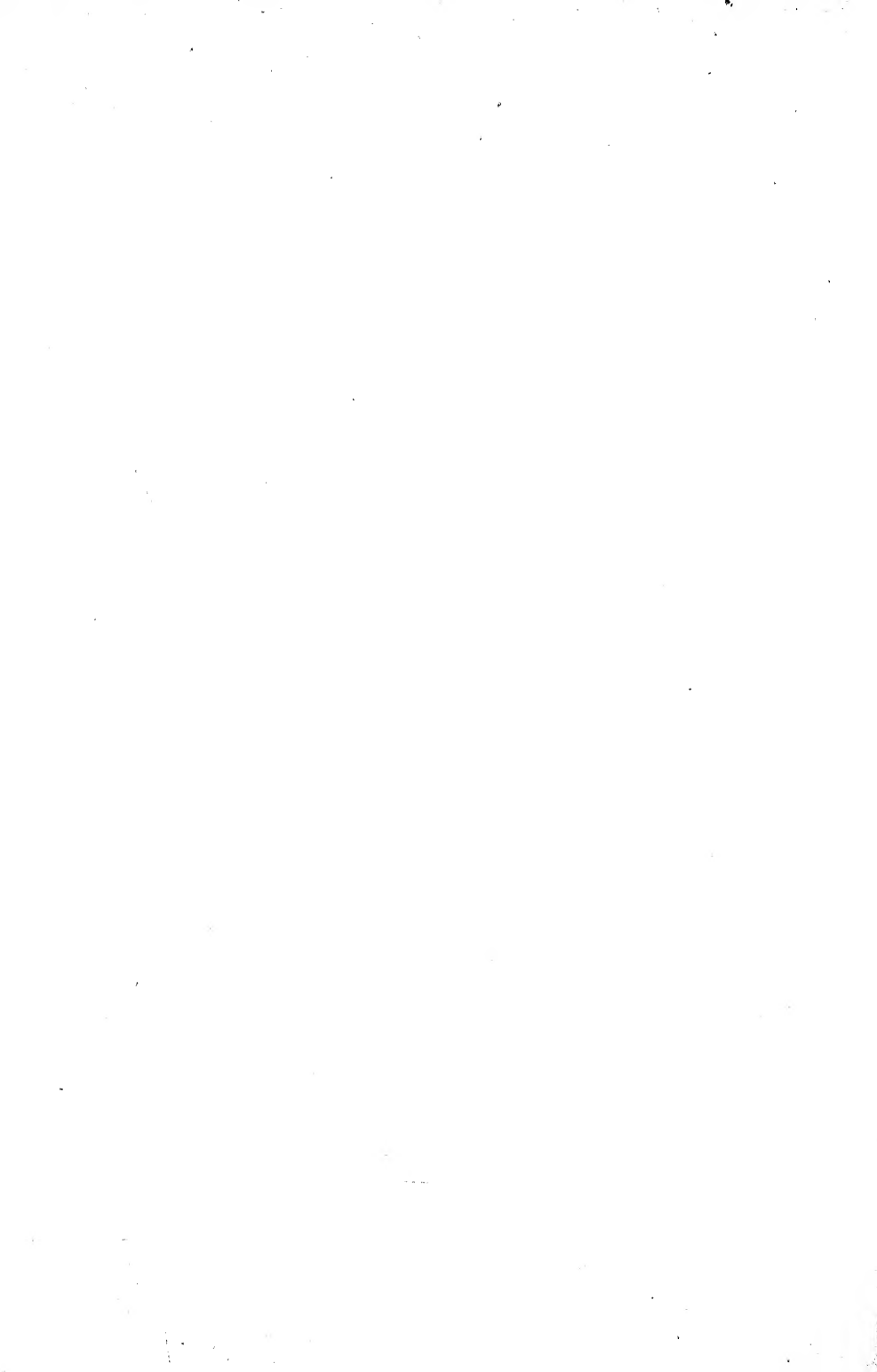
I am indebted to Dr. I. J. Lorch for assistance with the experiments, and to Prof. G. A. R. Kon and Prof. A. Hadow for their comments on the manuscript. The investigation has been assisted by grants made to the Royal Cancer Hospital by the British Empire Cancer Campaign, the Anna Fuller Fund, and the Jane Coffin Child Memorial Fund for Medical Research.

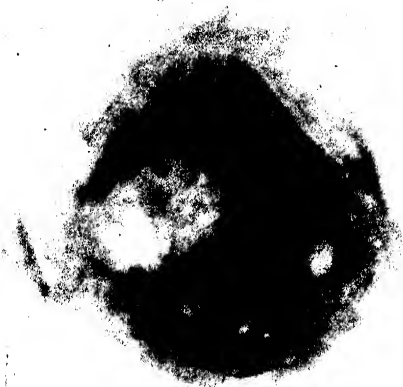
SUMMARY

1. During the absorption of fatty acids or of fat from the intestine of mice and rats, no intervention of long-chain aldehydes occurs.
2. When fat is being metabolized or synthesized in the liver of mice and rats aldehydes are prominent. Each fat droplet being metabolized is surrounded by a spherical shell containing aldehyde and basiphilic material.
3. The results indicate that long-chain aldehydes or their derivatives are essential intermediates in the metabolism of fatty acids, and suggest that the acetals of these aldehydes are intermediates in the synthesis of glycerides and phospholipins, &c.

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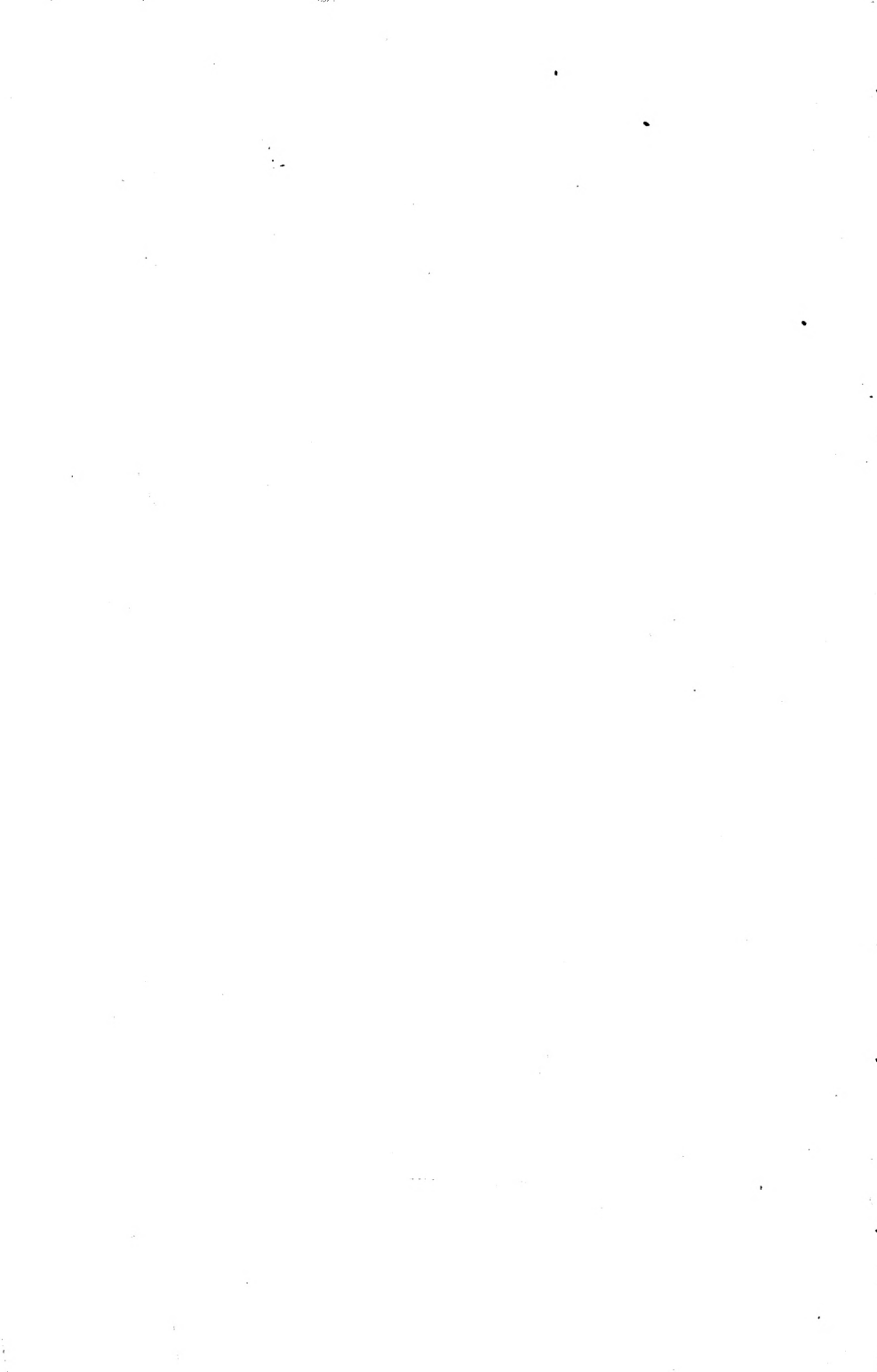
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Shows aldehyde in a single hepatic cell in a frozen section. The surrounding cells are aldehyde-free. The large clear central body is the nucleus. The shell of aldehyde appears as a ring surrounding a large fat droplet which is in focus. Another large fat droplet is out of focus, and there are many small droplets and some diffuse aldehyde

J. F. DANIELLI—PLATE I



The Histochemical Detection of Beryllium

BY

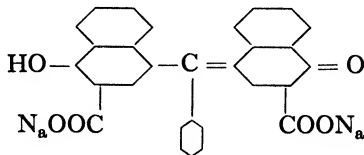
F. A. DENZ

(From the Experimental Station, Porton, Wilts.)

DURING an investigation of the toxic action of beryllium salts on experimental animals a histochemical test was developed to provide information on the distribution and site of action of beryllium. The technique is reported as an example of the application of chemical methods to histological problems.

The reactions of beryllium salts with dyes of the triarylmethane series have been studied by Aldridge and Liddell (unpublished) in the course of developing a more satisfactory method for the chemical determination of beryllium in biological material. From this work it appeared probable that the coloured lakes formed by beryllium and dyes of the naphthochrome series would be suitable for the detection of the element in histological sections. A small number of naphthochrome dyes were tested for laking properties with concentrations of beryllium varying from 1 to 10 μg . per ml. in phosphate buffers ranging from pH 4.5 to 9.0. The dyestuff naphthochrome green B at pH 5 forms with beryllium an apple-green lake that contrasts with the blue-black colour of the dyestuff. Iron and aluminium share with beryllium the property of forming lakes with naphthochrome green B, but these lakes differ in colour. The ferric lake is dark blue-green and the aluminium lake is yellowish-green in colour. Further, the optimum pH for lake formation differs for the three elements. The colour reaction between beryllium and naphthochrome green B is maximal at pH 5 but iron and aluminium at this pH produce very slight colour changes, and these only at relatively high concentrations. In this way by selecting the dyestuff and pH range, it is possible to find conditions which permit a characteristic colour change with beryllium, but inhibit any reaction with other elements such as iron and aluminium.

Naphthochrome green B (C.A.C.) has Schultz Number 851 and is the sodium salt of pheno-oxy-dinaphtho-fuchsondicarboxylic acid. Its structural formula is



Compounds of this type react with polyvalent metallic ions to form chelate compounds linking the metal to the adjacent hydroxy and carboxylic groups.

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It is probable that the coloured lake formed between naphthochrome green B and beryllium is produced by chelation.

METHOD

Two solutions are used.

1. 0.5 per cent. aqueous solution of naphthochrome green B. This must be freshly made immediately before use.
2. Phosphate buffer adjusted to pH 5.

PROCEDURE

Tissue is fixed in formol alcohol or formol saline, and passed through alcohols to xylene (or cedar wood oil) before paraffin embedding. The paraffin sections are treated with xylene and brought to water through alcohols.

Equal volumes of the naphthochrome solution and the phosphate buffer are mixed immediately before use. This solution does not keep more than a few hours. Stain slides for 30 minutes in a Coplin jar, the solution being maintained at 37° C. in an incubator during staining. At temperatures below 37° C. the intensity of staining decreases considerably. Wash in distilled water and differentiate by immersion directly in absolute alcohol for 30 minutes.

Wash in distilled water.

Counterstain with 1 per cent. aqueous solution of acridine red for 5 minutes.

Wash in distilled water, differentiate rapidly in absolute alcohol, pass directly to xylene and mount in Canada balsam.

The sites of beryllium deposits stain a clear apple-green against the red background of the counterstain.

BIOLOGICAL STANDARDIZATION OF THE TEST

Intradermal Injections and Skin Titrations

The method of intradermal injection was used to provide material containing known amounts of beryllium. Doses from 1 μ g. to 1 mg. of beryllium as 0.1 ml. of a solution of beryllium lactate were injected intradermally into the shaven backs of white rabbits. The lactate was chosen for this work, because the aqueous solution of this salt is near neutrality (pH 5.5) and the lactate ion is unlikely to cause any physiological disturbance. At intervals from 4 to 24 hours after inoculation the animals were killed and the injected areas were excised. The beryllium lake in the skin could be seen clearly in stained sections with inocula of 50 μ g. and the lowest detectable dose was 3 μ g. In addition to indicating the range of sensitivity, the skin titrations provided a rough set of colorimetric standards for comparison with tissues from animals injected intravenously with beryllium salts.

The same method was used to test the stain against the salts of iron and aluminium. In all comparisons of the staining properties of these salts and the salts of beryllium equimolar solutions were used. Solutions of ferric alum and aluminium sulphate were injected intradermally in white rabbits.

Subsequent staining showed that these elements could be detected only in concentrations at least ten times greater than beryllium. Furthermore, the almost complete absence of staining of tissues obtained 24 hours after inoculation showed that both iron and aluminium ions are rapidly removed from the site of injection. By contrast, beryllium remains at the site of inoculation in a stainable form for at least 3 months. It was concluded that iron and aluminium would not interfere because neither could be present in a concentration great enough to form a visible lake with the dyestuff. This conclusion has been confirmed by further animal experiment. Lake formation did not occur in the tissues of any control animals except in the pancreas, which stains a diffuse pale green colour.

Intravenous Injection in Animals

The size of the dose for intravenous injection and histological tracing was found by injecting the warmed tail vein of mice with doses of beryllium containing from 5 to 1,000 μg . of beryllium. The LD 50 for mice is 20 μg . of beryllium and it is naturally desirable that the tracing dose should be of this order. In animals killed 2 hours after injection strong staining was found following injections of 200 μg . of beryllium, but below this level the distribution of beryllium in the tissues was difficult to see. The level of 200 μg . of beryllium was chosen as optimal and the histological changes were followed up to 8 hours after injection, the mice being killed at regular intervals. Following injections of 200 μg . mice die within 24 hours, but after 50 μg . survive for 2 or 3 days. These later stages were followed in a further experiment at the reduced dosage. Fifty μg . was the least amount of beryllium that could be satisfactorily followed in the tissues of the mouse, even after considerable experience had been gained from the study of material from mice injected with 200 μg .

Utilization of the Method

The histochemical findings will be reported in detail elsewhere as part of an account of the toxicity of beryllium. An outline of the findings is given to illustrate the potentialities of such a method. By the naphthochrome method beryllium can be seen within the larger blood-vessels of all organs of the body for 15 minutes following intravenous injection of a soluble salt of beryllium. It then disappears from the general circulation and concentrates in the sinusoids of liver and spleen and in the vasa recta of the kidneys.

The development of the pathological lesions can be associated with the focal concentration of beryllium. In the liver where the overall concentration may be of the order of 5 μg . per gramme of tissue the focal concentration in the sinusoids is equivalent to 100–200 μg . per gramme. Around such concentrations the liver cells are destroyed and the characteristic focal necrosis of beryllium poisoning develops.

Beryllium in tissues and in blood appears to be attached to protein. This union accounts for its persistence at the sites of injection, and the sharpness

with which it stains. The formation of focal concentrations within sinusoids is a most important factor in enabling the tracing to be carried out. Fifty $\mu\text{g.}$ of beryllium uniformly dispersed throughout the tissues of a mouse would produce a concentration of about 2 $\mu\text{g.}$ per gramme and this is below the limits of histochemical detection by the naphthochrome method. However, following the injection of 50 $\mu\text{g.}$ of beryllium focal concentrations within liver, spleen, and kidney reach values fifty times as great as this and are readily seen.

Pathological change follows focal accumulation of beryllium and a combination of the naphthochrome method and routine histological methods has enabled such problems as the development of midzonal liver necrosis and circulatory changes in the kidney to be studied.

LIMITATIONS AND DIFFICULTIES

Effect of Solubility of Beryllium Compounds

The test is specific for beryllium but the dyestuff does not react with all compounds of beryllium. The refractory oxide and the silicate are insoluble in water, resist the action of acids, and do not react with naphthochrome green B. *In vitro* lake formation with naphthochrome green B occurs only with the beryllium ion but the situation in tissues is more complex. If dilute solutions of beryllium salts are mixed with serum the beryllium cannot be separated from the serum proteins by ultrafiltration and in general beryllium ions appear to combine rapidly with the tissue proteins. This union is firm enough to retain beryllium at the site of subcutaneous injection for months but does not prevent the reaction between beryllium and naphthochrome green B.

If the refractory oxide or the silicate is injected into the skin, solution of the injected material takes place very slowly so that after some weeks faint green staining of the inoculation site is obtained with naphthochrome green B. This occurs only when the inoculum is large and the tissue reactions have been going on for a long time. When the insoluble compounds are widely dispersed in the tissues after inhalation of dusts or following the intravenous injection of suspensions the histochemical test does not disclose the localization of the oxide or silicate particles. The test is of value only for the study of the reaction of tissues to the injection of soluble compounds.

Dosage Level

The optimum tracing dose was found to be several times the LD 50 and one of the difficulties, that of early death of the animal, has already been mentioned. A further difficulty is evident when the possibility that the distribution of the higher doses in tissues may be different from that of lower doses is considered. This is certainly the case in the mouse lung. At an inoculation level of 200 $\mu\text{g.}$ of beryllium the lungs stain strongly and the staining persists for 8 hours at least, whereas with 50 $\mu\text{g.}$ staining is never diffuse, is always slight and entirely disappears within an hour of injection. Again beryl-

limum was present in the medulla of three out of four adrenals from animals injected at the higher and only in one of twenty-three at the lower level. In this investigation the assumption was made that where the distribution of beryllium within the organs was constant in a large series of animals at both levels of injection, i.e. at 200 and at 50 $\mu\text{g.}$, this distribution was likely to be present at even lower levels. This assumption was tested against pathological findings and the results of chemical analysis and was not found to be incorrect.

Contamination

Owing to the sensitivity of the staining method contamination is a factor that must be considered in the handling of histological material. Contamination by iron, aluminium, or beryllium salts must be avoided. Fine specks of rust give an intense colour reaction. Care must be exercised during dissection of the injected animal. If the beryllium-rich liver is cut before tissues such as thymus or muscle which do not contain beryllium, sufficient may be carried over on the knife to give a considerable degree of diffuse staining in the latter organs. A fresh knife should be used for each organ. The tissues may, however, be fixed in a common pot.

Artifacts

Those due to contamination have been mentioned above. The staining is often more intense at the margin of the section than at the centre. Occasionally the margins of control and experimental tissues are stained a diffuse green. This is usually but not always due to overlong staining, and in some cases the cause has not been found. The artifact is sufficiently obvious to prevent confusion with the staining of the beryllium within the tissues. The exocrine tissue of the pancreas usually takes on a blue-green tint that is not extracted by alcohol. This colour is slight and diffuse and readily distinguished from the focal apple-green staining of beryllium deposits.

ACKNOWLEDGEMENTS

This work owes much to the help and encouragement of Dr. J. M. Barnes and Mr. W. N. Aldridge who have been so closely associated with it that I find it impossible to separate their contributions and mine. I wish to acknowledge the assistance of Mr. H. F. Liddell whose exceptional knowledge of dyestuffs made this work possible.

SUMMARY

A histochemical test for beryllium in paraffin sections is described. A green lake that is formed with beryllium by the dyestuff naphthochrome green B is specific for beryllium under the stated conditions. The test works only with the soluble compounds of beryllium. Deposits of as little as 3 $\mu\text{g.}$ of beryllium can be detected and the distribution in the tissues of mice can be followed after the intravenous injection of 200 $\mu\text{g.}$ of beryllium. The applications and limitations of the method are discussed.



A Simple Method for Phase-contrast Microscopy: Improvements in Technique

BY

JOHN R. BAKER, D. A. KEMPSON, AND P. C. J. BRUNET

(From the Department of Zoology and Bureau of Animal Population, Oxford)

IF the condenser of a microscope is first set in its so-called 'critical' position and then gradually lowered, the real image of the source of light will be brought down from the plane of the diaphragm of the eyepiece till it reaches a position just behind (above) the back focal plane of the objective. If a piece of black paper, from which a ring-shaped strip has been cut away, is held immediately in front of the source of light, a bright image of the annulus can easily be thrown just behind the back focal plane of the objective by lowering the condenser to the appropriate degree. If now a phase-plate, provided with an annulus, is placed in the same plane as the image of the illuminating annulus, and if the phase-plate annulus is of such dimensions that it coincides with this image, phase-contrast microscopy will have been achieved.

This method of phase-contrast microscopy was suggested by one of us in a paper on oblique illumination (Baker, 1948). It was worked out practically by Kempson, Thomas, and Baker (1948). Full instructions were given on the adaptation of an ordinary microscope for phase-contrast work. The method requires no special objectives and no special apparatus for centring the illuminating annulus. It is therefore extremely simple and inexpensive.

The purpose of the present paper is to record certain improvements in the method. Still further simplification has been achieved, while the results obtained are better than before.

In the earlier paper we gave exact instructions for using phase-contrast with a Zeiss apochromatic water-immersion objective. We now think it best to give instructions for using phase-contrast with lenses that are readily available in this country. We have confined ourselves to the products of Messrs. W. Watson, as follows:

Objectives

- 4 mm. (1/6th inch) 'Parachromatic', N.A. 0.7, dry,
- 4 mm. (1/6th inch) 'Parachromatic', N.A. 0.8, dry,
- 1.8 mm. (so-called 1/12th inch) 'Versalic', oil-immersion.

Condensers

- Ordinary two-lens Abbe,
- 'Parachromatic'.

Full details of the proper sizes for all the parts of the apparatus, when these lenses are used, are given in Table 1. It was thought convenient to collect all the numerical data in a single table, so that they need not be given elsewhere.

The reader is referred to the earlier paper for a general account of the method adopted. In the present paper we restrict ourselves to the improvements we have made since that paper was written.

Illuminant. We have found the best illuminant to be a powerful electric bulb with a 'porcelain-processed' surface. 'Flashed-white' bulbs are quite good; 'opal' ones have the advantage of a very homogeneous surface, but unfortunately this absorbs much of the light, so that they are not suitable for high-power work. 'Pearl' bulbs are inferior, and coarsely frosted ones are to be avoided. The best bulb known to us is the 150-watt 'Helios' porcelain-processed enlarging bulb, intended for use in photographic enlargers. These are made with the whole surface or the tip alone processed. We use the bulb made for 200–220 volts, but overrun it on the mains at 230 volts. We no longer use a bull's-eye condenser.

Illuminating annulus. The simplest way of making this is to cut an annular strip out of a sheet of black paper, and to glue both the central and the outer part of the sheet on to a piece of plain glass in such a way as to leave an annular space of uniform width between them. However, the heat from the electric bulb may crack the glass and we therefore prefer to use brass. The central disk is fixed to the outer piece by four narrow radial spokes, which traverse an annular empty space and are soldered to the brass at each end. The illuminating annulus should be placed close to the electric bulb.

The appropriate sizes for annuli (outer and inner radii) are shown in Table 1. The size necessary to produce coincidence between the phase-plate annulus and the image of the illuminating annulus is dependent on five factors: (1) the distance of the illuminating annulus from the condenser (via the mirror); (2) the focal length of the condenser; (3) the focal length of the objective; (4) the distance of the phase-plate from the back focal plane of the objective; and (5) the radii of the phase-plate annulus. The image of the illuminating annulus should theoretically coincide exactly with the phase-plate annulus. However, any straying of the image of the illuminating annulus beyond the limits of the phase-plate annulus is very damaging to contrast, while the reverse causes little deterioration in the image. For this reason we generally make our illuminating annulus of such dimensions that the image of it is slightly narrower than the phase-plate annulus, the edges of which can be seen outside and inside the image of the illuminating annulus.

The first image of the illuminating annulus (below the object) is a long way from the aplanatic plane of the objective, and the second image (in the plane of the phase-plate) is therefore imperfect: that is, the outer and inner edges of the image are not in exact focus at the same time. We do not find that this causes any observable detriment to the image of the object as seen through the eyepiece of the microscope.

TABLE I

Objective				Condenser			Phase-plate			Illuminating Annulus			Column Number	
Name	Focal length	N.A.	Radius of back lens	Name	Focal length	N.A.	Distance above back lens of objective	Total radius	Radii of annulus		Distance from bottom lens of condenser†	Radii		
									Outer	Inner		Outer		Inner
Parachromatic	4	0.7	2.6	Abbe	12	1.2*	1.1	3.0	1.0	0.7	160	21	16	1
"	"	0.8	2.9	"	"	"	4.1	3.5	1.1	0.75	180	26	18.5	2
"	"	"	"	Parachromatic	7	0.9	"	"	"	"	190	"	"	3
Versallic	1.8	1.28	2.25	"	"	"	2.2	2.75	0.9	0.63	210	19	15	4
"	"	"	"	"	"	"	0.3	2.38	"	0.7	190	"	"	5
"	"	"	"	Abbe	12	1.2*	"	"	"	"	280	21	16	6
"	"	"	"	"	"	"	2.2	2.75	"	0.63	360	19	15	7

All linear measurements are given in millimetres.

The objectives and condensers listed in this Table are made by Messrs W. Watson.

The annuli of the phase-plates are generally smoked so as to allow the transmission of 75 per cent. of the light falling upon them.

* Not aplanatic.

† Via the centre of the mirror.

Mirror. A stainless steel mirror, or any other kind that gives only a single reflection, is slightly preferable, but an ordinary microscope-mirror gives satisfactory results.

Colour-filter. A filter, giving maximum transmission at $500\text{m}\mu$, should be used when photographs are taken. For visual work we generally prefer to use no filter, since to the human eye the yellowish light of the electric bulb gives a better contrast against grey and black than does green, and this appears to us generally to outweigh the optical advantages of illumination with a wave-band closely adjusted to the thickness of the magnesium fluoride on the phase-plate.

Condenser. The chromatically and spherically corrected Parachromatic condenser presents two advantages over the Abbe: objects are seen without any tendency to adventitious colour, and the phase-effect is obtained right up to the edge of the field of view. When the Abbe is used, the phase-contrast is not good near the edge of the field of view. Nevertheless, quite good results are obtained with the Abbe, and no one should be dissuaded from using the method simply because he has not got a corrected condenser.

Phase-plate holder. We no longer use spring jaws, but simply cement the plate in the holder. The inner surface of the holder must be blackened.

Phase-plate. The radius of the phase-plate must be at least as great as that of the black lens of the objective.

If the back focal plane of the objective lies within its lens-system, the phase-plate should be placed close behind the back lens. If the back focal plane of the objective lies behind (above) the back lens, the phase-plate should be placed a millimetre or two behind (above) this plane.

The thickness of the magnesium fluoride film deposited on the lower surface of the phase-plate should be such that it retards light to the extent of approximately a quarter of a wave-length of apple-green light, in comparison with light travelling through a film of air of equal thickness. (Apple-green light is chosen because it lies near the middle of the spectrum of the light used for illumination.) Our phase-plates are optically flat circles about a millimetre thick. They are made by Messrs. R. & J. Beck, who also deposit the magnesium fluoride upon them. Care should be taken to check the thickness of the deposit. This can be done by holding the plate over a dark background and looking at it in such a way that the line of vision subtends an angle of approximately 30° to a line perpendicular to the surface of the plate. If the retardation is exactly $\frac{500}{4}\text{m}\mu$, the interference colour seen in daylight at this angle will be blue-green (in the second order of Newton's rings). Our experience is that the very best results are obtained when the film is slightly thinner than this, so that the colour given is blue.

In our earlier paper we described the making of a 'negative' or bright-contrast plate, that is, a plate in which the annulus consists of a *wall* of magnesium fluoride standing up from the glass. We now find that for nearly all objects 'positive' (dark) phase-contrast is preferable. The annulus is there-

fore excavated in the magnesium fluoride to the level of the glass, and exists as a *ditch* instead of a wall. To measure and mark off the annulus to be cut in the coating, a micrometer eyepiece should be slipped into the binocular used for observing the cutting operation (or into a monocular with a suitable low-power objective). The diameter of the optical flat being known, the eyepiece-scale can be calibrated accordingly and the boundaries of the annulus marked with a finely pointed needle. Owing to the small amount of scraping required to make a 'ditch' for a positive phase-plate, the method previously given for operating the tool in making a negative plate was abandoned in favour of a tool-rest. This is simply a $\frac{1}{4}$ -inch rod, held horizontally and supported at each end by convenient fixtures on the bench. The rod is placed close to the phase-plate on the turntable, so that the scraper, held in the hand, rests in a V-mark filed in the rod. A rigid support is thus provided, yet freedom of movement is permitted. Care must be taken not to mark the glass with the tool by continuing to scrape longer than is necessary. Progress is easily observed if the lighting is adjusted to the correct angle and the plate frequently wiped with a grease- and grit-free handkerchief. The difference in the coated surface and the clear glass is unmistakable.

We have had our best results with phase-plate annuli of the sizes shown in Table 1. It will be observed that the annulus is placed nearer to the centre of the plate than to an imaginary circle drawn round it, equal in diameter to the back lens of the objective. This is a purely empirical finding.

The amplitude of the direct (undiffracted) light is reduced by smoking the plate over a flame and then removing the smoke from the whole surface except the annulus. For any particular object there is an ideal degree of reduction of amplitude of the direct light. In general, to suit a wide range of objects as well as possible, we find that the smoking should be of such a depth as to allow the transmission of 75 per cent. of the light reaching the annulus. The fuel used for making a smoky flame is a mixture of absolute alcohol and xylene, the latter being added until the flame gives off a slight smoke. Too much xylene causes a coarse deposit of carbon and produces irregular and streaky effects.

We nowadays generally check the depth of smoking by the use of a step-wedge. The comparison with the step-wedge is made while the whole surface of the plate is still covered with smoke. If a suitably graduated step-wedge is not available, a simpler and convenient standard can be made from Ilford 35 mm. Leica film or Kodak Super XX 35 mm. film. When the emulsion is cleaned off, the grey-tinted base has a transmission of from 60 to 70 per cent. The desired amount of smoking being 75 per cent. for the phase-plate, it is evident that the smoke density on the phase-plate should be a trifle lighter than that of the film base. The wedge or film base should be masked so that only an area of the same size as the smoked phase-plate is used for comparison.

Auxiliary microscope. The low-power lens used for centring and focusing the image of the illuminating annulus with the phase-plate should have a low numerical aperture. A cardboard stop should be inserted in the lens system if necessary.

It may be found convenient to use a special auxiliary microscope, fitting in place of the eyepiece. In this auxiliary microscope the objective is the field-lens of a low-power eyepiece, with a cardboard stop about one-third its diameter immediately above it; the eyepiece is *either* the whole *or* the eye-lens only of an ordinary high-power eyepiece. A brass tube 6 cm. long, split longitudinally, will hold the 'objective' and eyepiece together in their tubes and permit focusing movement.

This method is particularly valuable when a binocular microscope is used.

Miscellaneous remarks. Columns 4 and 5 in Table 1 are alternatives; so are columns 6 and 7. The phase-plate used in columns 5 and 6 is held very close to the back lens of the objective, and it is rather difficult to make a cell to hold it in this position. The cell for the phase-plate described in columns 4 and 7 is easier to make, but the results obtained are probably not quite so good.

On changing from a medium-power to a high-power objective, it is generally necessary to move the lamp, so as to get the correct distance between the illuminating annulus and the lower lens of this condenser. It will be found convenient to make marks on the bench, so that the lamp may be quickly placed in the right position.

Cleanliness is even more important than in ordinary microscopy. Any dirt on the top lens of the condenser *or on the bottom of the microscopical glass slide carrying the object* is likely to divert some of the direct light so that it falls elsewhere than on the annulus; this necessarily reduces contrast.

The preparation should be as thin as possible; that is to say, there should be no unnecessary fluid or mounting medium between slide and coverglass.

We claim for this method of phase-contrast microscopy that it is at least as good as the ordinary method, much less expensive, and applicable to any microscope.

ACKNOWLEDGEMENTS

Dr. O. L. Thomas's collaboration only came to an end when he left this country for New Zealand. We had already discussed with him our proposals for attempts to improve the method.

Professor A. C. Hardy, F.R.S., has continued to give every possible support to our work. We have received valuable advice on matters of theory from Dr. H. Kuhn and Dr. A. G. Oettlé. Mr. A. R. S. Bell has rendered us particularly skilful practical assistance.

SUMMARY

The following are the main improvements that we have made in the method of phase-contrast microscopy described by Kempson, Thomas, and Baker (1948):

1. No bull's-eye condenser is used. The illuminant is an electric bulb with a 'porcelain-processed', 'flashed white', or 'opal' surface.
2. No oiled paper is placed over the illuminating annulus.

3. The thickness of the deposit of magnesium fluoride on the phase-plate is controlled by observations on the interference colours given by surface reflections.

4. Positive (dark) phase-contrast is preferred for most purposes to negative (bright).

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The Cell-Theory: a Restatement, History, and Critique

Addendum to Part II

BY

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SINCE writing Part II of this series of papers (Baker, 1949), I have had the good fortune to find a figure, published by Roffredi in 1775, which shows nuclei in the eggs and embryos of a nematode. This figure antedates by fifty-five years Purkinje's description of the germinal vesicle (1830) (or by fifty years, if we use the date of the private circulation of Purkinje's memoir).

Roffredi (1775) describes the anatomy of a small nematode obtained by wrapping flour-paste in linen and leaving the parcel in earth. It is evident from the drawing (Fig. 1 on the plate placed before p. 297) that the animal is a female belonging to the genus *Rhabditis*. The anterior and posterior ovaries and the eggs and young embryos are shown.

Roffredi regarded the whole of the female system as the 'ovaire ou matrice', and called the true ovaries (or ovaries and oviducts) its 'extrémités'. Nuclei are clearly represented in the oocytes that lie in a row along each ovary, and also in the eggs and embryos. Mutual pressure in the ovaries gives each of the oocytes a squarish appearance, and he calls the cell and its nucleus 'le quarré et le globule'. He does not give a written description of the nucleus in the mature egg nor in the embryo.

Spots are shown in some of the nuclei, but there can be no certainty that any of them represent nucleoli, and it seems best to continue to credit Fontana (1781) with the discovery of this organelle.

Roffredi unfortunately failed to distinguish the boundaries of the blastomeres. It is tantalizing to consider how easily he could have discovered cleavage if he had followed up his observations.

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On the Functional Morphology of the Alimentary Tract of Some Fish in Relation to Differences in their Feeding Habits: Cytology and Physiology

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With four Plates

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HISTORICAL INTRODUCTION

THE present paper forms the continuation of work already reported in an earlier paper (Al-Hussaini, 1949). The same three cyprinid fishes have been used for comparative study, namely, the mirror carp, *Cyprinus carpio* as an example of a herbivore, the roach, *Rutilus rutilus* as an omnivore, and the gudgeon, *Gobio gobio*, a predominantly carnivorous fish.

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Much of the general literature concerning the alimentary canal of fishes has been reviewed in Part I of this work and only those papers directly bearing on the subject under review will be mentioned. Amongst the earliest workers to investigate the physiological aspects of the fish gut were Tiedmann and Gmelin (1827), while half a century later Homburger (1877) studied digestion in several species of carp, and reported that the bile and extracts of the liver as well as of the intestinal mucosa were capable of digesting fibrin, emulsifying fats, and of hydrolysing starch to sugar. Krukenberg (1877-8) concluded that, although no fish possesses salivary glands, some, as *Cyprinus carpio*, have a diastase in the mucous membrane of the mouth, while in some teleosts, such as *Cyprinus* and *Gobius*, the organ occupying the position of the stomach secretes no enzymes at all, digestion being effected in the middle intestine. He found trypsin in this region as well as in the liver in cyprinids. In contrast to Krukenberg, Luckhau (1878) found no enzyme which would digest albumen. He also failed to find a lipase or any proteolytic enzyme working in an acid medium (pepsin). He did, however, find an amylase and a proteinase working in a neutral or alkaline medium (trypsin) in various parts of the intestine. Blanchard (1882) found an amylase and a lipase in the rectal gland of elasmobranchs and an amylase and a proteinase in the pyloric caeca of teleosts, which structures he regarded as representing a pancreas. The latter finding was confirmed by Stirling (1884) working on the herring, cod, and hake.

Kenyon (1925) studied the digestion of carbohydrates and proteins in cold-blooded vertebrates comparatively, and contrasted the processes with those of a mammal (dog). Amongst his examples of teleosts he used the common carp. He concluded that little change in the general character or rate of activity of enzymes has occurred during the evolution of vertebrates but that a close correlation exists between the morphology of the gut and the distribution of enzymes (e.g. no pepsin in the stomachless carp). He demonstrated erepsin for the first time and showed certain adaptive features of enzymes to the diet (e.g. more amylase in herbivorous than in carnivorous species). Vonk (1927) studied the range of temperature and pH over which fish enzymes (including those of the common carp) are active, and their respective optima. He found the digestion of fishes similar in most respects to that of mammals. In 1937 he reviewed the specificity and collaboration of digestive enzymes in the Metazoa.

The more specialized cytological and histochemical literature is very recent and it will be more convenient to deal with this at the relevant places in the text, as there are very few previous observations on fishes and most of the references involve comparative discussion.

MATERIAL AND METHODS

The material used in the present study came from the same sources as that used for the anatomical and histological study (cf. Part I). Numerous techniques were used, many of them standard procedures, but some required

special modification. It will, therefore, be more convenient to refer to such technical methods in the appropriate sections of the text.

I. THE CYTOLOGY OF THE INTESTINAL EPITHELIUM

A. The Absorptive Cells

1. The Free Border

Baker (1942) based his study of the free border of the intestinal absorptive cells of vertebrates on tetrapods and made no mention of fishes. In a personal communication to the writer he said that he examined two fresh-water fishes only, and that their free border was simpler in structure than that of his tetrapod types. Baker's methods have therefore been applied to the present three cyprinids with the object of discovering any possible deviation from the general plan which he described as common to all the tetrapods he had examined. The crested newt, *Triturus palustris*, was used as a control. Material from the three Red Sea fishes studied earlier (Al-Hussaini, 1945, 1946, 1947) was also reinvestigated from this point of view.

For a full history of the literature dealing with the luminal edge of the absorptive cells of vertebrates reference should be made to Baker's review, but, briefly, it may be said that there are two theories; the one claims that the border consists of cilia or rods, and the other that the border is traversed by minute canals which open at the surface as pores. Baker adheres to the second alternative (cf. Baker's text-figs. 1 and 2).

In the cyprinid absorptive cell the free border is built up of three principal layers (Pl. I, fig. 1). These are the superficial layer (S.F.L.), the canal layer (C.L.), and the granular layer (G.R.L.). Immediately deep to the free border, within the cytoplasm of the cell, is the subgranular layer (S.G.L.), and this, in turn, is separated from the supranuclear group of mitochondria (S.P.M.) by a clear zone (C.L.Z.).

The free-border in the cyprinids is rather thinner (about 2.5μ) than that of the newt. The superficial layer is best seen in living cells examined in isotonic saline, or in iodized serum, or in preparations fixed and mounted in 4 per cent. formaldehyde; in other words, in aqueous mounted material. It appears as a very thin strip extending along the entire surface of the free border, which, on careful focusing, is seen to display a beaded appearance rather than a uniform thickness; nevertheless very few openings of the canals were encountered. In clarite mounted material the superficial layer is usually obscured, but in two instances it was seen in material (from *Rutilus* and *Cyprinus*) fixed in M.A.S. of Baker and stained with Heidenhain's haematoxylin.

The canal layer can be demonstrated by several techniques, ordinary routine ones as well as those intended for special purposes, e.g. Kull's technique for mitochondria, or Ciaccio's for lipines (Pl. I, fig. 2), although Baker claims that fixation in M.A.S. followed by acid violet yields the best result with his material. The canals may even be seen in the living cell as faint striations. The canal layer stains light green with Masson's trichrome stain, indicating,

as Baker suggested, that it contains collagen. The superficial layer stains an even more intense green by this method. The canals usually appear spindle-shaped, although sometimes they appear constricted in the middle; they are narrower and fewer in number than in the newt.

It is uncertain whether the structures revealed by routine methods are the canals themselves or optical sections of the substance between the canals. The striking appearance of the canal layer has resulted in some reference to the free border appearing in almost every work on the histology of the fish gut, although it is described under various names, e.g. 'striated border', 'brush border', 'top plate', or 'cuticle'. Some writers have described cilia in various places in the intestinal epithelium of teleosts, such as at the entrance to the pyloric caeca (Edinger, 1877), within the pyloric caeca (Rahimullah, 1945), or along a very short tract of the long intestine of *Salarius enosimae* (Ishida, 1935). It is possible that the structures described as cilia are really the intercanal substance in a border unusually thick.

At many places between two adjacent cells there is a canal which, while still fine, is larger than the canals just described and which opens freely to the surface by a distinct pore. Baker considers this canal to be an artifact due to an interruption of the border and not a distinct passage; nevertheless, its frequent and persistent occurrence makes one sceptical about its artificial nature and I am more inclined to regard it as a real structure offering permanent passage into the cell, although one must admit that it cannot be found at every cell junction.

The granular layer appears as a dark line in the living cell but shows no granulation, and this may also be true in the fixed cell when treated by some methods, e.g. by Baker's modification of the Smith-Dietrich method for lipines, or with Schulze's chlor-zinc-iodine, although in the latter case the dark line shows a slight intermission. If the sections are given a preliminary treatment with diaphanol before applying Schulze's method, the granular layer becomes somewhat swollen, but no spherical granules such as those described by Baker could be seen.

The subgranular layer and the clear zone are distinct both in the living cell and in material fixed in M.A.S. In sections specifically prepared to demonstrate mitochondria the clear zone is especially distinct.

The intercellular band (Pl. I, fig. 1, 1.B) may be easily observed in the living cell and stands out clearly in material stained by Heidenhain's haematoxylin (Pl. I, figs. 3 and 5), while with mitochondrial techniques the band takes the same colour as the mitochondria and the granular layer. It is in the form of a rim or girdle which encircles the edges of the cells approximately at the level of the granular layer (its precise position may be seen in Pl. I, fig. 1) so that in tangential sections the band appears as a polyhedral or hexagonal outline like a honeycomb (Pl. I, fig. 3). Its deep edge merges imperceptibly into the intercellular membrane. Baker described the band in tetrapods as 'usually wider than high', but in the cyprinid gut its dimensions are reversed.

When two limbs of adjacent folds come into direct contact with each other the border appears much thicker than usual and the canals appear stretched (Pl. I, fig. 4), suggesting that the free border is elastic and capable of expansion and that this has been effected by the temporary adhesion of the free surfaces, so that, when the muscularis layer contracts during the normal peristaltic movement of the intestine, the free border is slightly stretched. On the other hand, the reverse may occur, and the free border may appear in places to be very much contracted so that none of the details just described may be seen. It follows therefore that the border may, and probably does, undergo a series of changes during the absorptive processes, and it is by no means impossible or even improbable that these changes may assist the passage of digested food into the cell.

In *Scarus sordidus*, the free border is of the 'brush border' type. In the anterior unsacculated portion of the intestine (duodenum) the border is particularly deep (4.5μ against $2-3\mu$ for the remainder of the gut) and the canals are sometimes constricted in the middle and sometimes widely separated. The passages between adjacent cells are clearly seen and the border appears to consist of 'clutches' of canals, each 'clutch' belonging to one absorptive cell (Pl. I, fig. 7). In *Mulloides auriflamma* and *Atherina forskali* the free border is similar to that of the cyprinids.

A species of chicken-fish, *Pterois volitans*, has a peculiar free border. It is strikingly thick ($7-11\mu$) and along the bases of the crypts, where the absorptive cells are shortest, the border accounts for about one-third of the total length of the cells. It appears as though consisting of interlacing fibres leaving between them distinct passages, sometimes of considerable calibre, presumably equivalent to the canals of other fishes (Pl. I, fig. 6).

The free borders described here can be arranged in a series which might perhaps have an evolutionary significance, showing the transition from the ciliated epithelium of lower chordates to the typical free border described by Baker in tetrapods. In such a series *Pterois* would be the most primitive, the duodenal epithelium of *Scarus* intermediate, and the remaining species the most specialized.

2. The Mitochondria

Numerous methods were employed for this section of the work, but it was found that Kull's method following fixation in Helly's fluid and post-chroming at 37°C . for 2 days (cf. Volkonsky, 1928, and Baker, 1945) was superior to all the rest.

The mitochondria are arranged in two groups, as in mammals; both contain rod-like as well as granular chondriomes (Pl. I, fig. 1), but the rod-like form is commoner in the supranuclear group and the granular type is more frequent in the subnuclear zone which extends into the basal processes of the cells and is better developed in *Gobio* than in *Cyprinus* or *Rutilus*. Indeed, the mitochondria are on the whole coarser in the carnivorous gudgeon than in the other two fish, but this difference may well be due to specific causes

rather than to the nature of the fish's food, since these organoids are subject to great variation in different cells even of the same fish.

Concerning the function of the mitochondria in the intestinal epithelium, Champy (1911-12) found that, in the mammal, during digestion and absorption the mitochondria in the cells of the intestinal villi become thread-like in form and less numerous. He suggested that their bipolar distribution is associated with the dual function of secretion and absorption. Liu (1930) working with mice found that the intestinal mitochondria show no fusion or decrease in number during carbohydrate absorption, but that during fat absorption they change rapidly within the first 6 hours after feeding the animal on fat from the rod-like form to fused masses. Cowdry (1924) also found a progressive decrease in mitochondrial numbers with an increase in the number of fat globules, as did Bourne (1942). Duthie (1935) showed that surviving liver cells, soon after transplantation, often store fat in large quantities and that during this process the mitochondria become short rods and lie in close relation to the fat droplets, but, on the other hand, Cramer and Ludford (1925) maintain that mitochondria do not appear to take any active part in fat absorption.

Experiments with the present cyprinids have shown that 3 days after an animal diet, while fat absorption is still going on but when the gut lumen is free from undigested food, the mitochondria of the absorptive cells are less numerous than they are when the gut is resting. The globules of absorbed fat may be found scattered throughout each absorptive cell except in the clear zone and for a short distance below it; those lying in the luminal portion of the cell being larger than those towards the base. Each fat globule appears red-edged which, on critical examination under oil immersion, is seen to be due to the acid fuchsin taken up by the mitochondria, thus suggesting that these organoids do have a close association with fat absorption. Confirmatory evidence was obtained by giving a small fish (about 10 cm. long) a meal rich in fats through the mouth and killing it 4 hours later. Abundant fat globules had already passed into many absorptive cells but not into all of them, thus making it very easy to compare the disposition of mitochondria in active with that in inactive cells within the same fish. The active cells showed a picture similar to those of the fish killed 3 days after feeding. The mitochondria had already undergone a rapid disintegration and lost much of their rod-like shape. The inactive cells which had not absorbed any fat resembled those of a fasting fish. In a second experiment four fasting roach of about equal size were each given a meal in semi-fluid form of different composition. The first was a suspension of starch, the second one of casein, the third a fat emulsion, and the fourth an inert substance (magnesium trisilicate in liquid paraffin). The fish were killed 4 hours afterwards. Subsequent examination showed practically no change from the resting condition in the mitochondria of the absorbing cells of the first and fourth fish, those of the second fish showed only a slight diminution in their numbers, but the third fish gave a picture similar to that of the fat-fed fish described above. The close associa-

tion between mitochondria and fat absorption in fishes, as in mammals, seems therefore to be well substantiated.

3. The Golgi Element

Baker (1944), in his valuable contribution to our knowledge of the structure and chemical composition of the Golgi element, concluded that it consists essentially of four parts, namely, 'neutral red vacuoles', 'dense lipid-containing substance', 'diffuse lipid-containing substance', and 'Golgi product' (cf. Baker's figures on pp. 24-5 of his paper). He used various types of cells for his study, including the absorptive cells from the intestine of the newt. Baker contends that the classical methods used to produce the Golgi network cannot be relied on to give an accurate picture of the structure of the element during life, and accordingly he introduced a new procedure—the formol-Sudan-black technique. In the present study this new technique, several osmium and silver impregnation methods, as well as intra-vitam stains were used (*vide infra*).

When the intestinal epithelium is examined in the living condition in saline in a compressorium the vacuoles of the Golgi element are clearly seen, and are readily stainable intra-vitam by neutral-red as described by Baker or by methylene blue by the method of Worley (1943). The majority are spherical but are variable in size from cell to cell or even in the same cell and their arrangement follows no definite pattern. Some three to five vacuoles are found in each cell.

In fixed preparations the Golgi element always lies in the apical part of the cell on the luminal side of the nucleus. Corti (quoted from Macklin and Macklin, 1932) maintains that in the gastric glands of the frog and mouse it may be either supra- or subnuclear, but it has never been seen in a subnuclear position in the cells of the cyprinid gut. It may be separated from the nucleus by a short interval or it may abut closely on it to form a sort of cap (Pl. I, fig. 1, G.E). With Baker's formol-Sudan-black technique a few of the cells showed the Golgi element clearly, but in the majority it appeared only as a dark area. The vacuoles were sometimes visible and in addition thread-like structures could be detected. They lie more or less parallel with the longitudinal axis of the cell, but are not quite straight. Incidentally, it should be mentioned that both groups of mitochondria and the granular layer of the free border of the absorptive cells as well as the intercellular band were all stained blue-black by this technique but less intensely than the Golgi element.

The dense lipid-containing substance is impregnated strongly by Kolatchev's method and appears as two or three black threads enclosing a number of vacuoles, varying in size and number, and hardly separated by any intermediate substance (Pl. II, fig. 9) and without showing the Golgi product (cf. Baker, 1944). A comparable result is obtained by Weigel's technique and also by Nassonow's method except that in the latter case the dense lipid-containing substance is more compact, the whole element appears contracted,

and, except in a few cells, the vacuoles are not so distinct. The osmium impregnation method which best preserves the vacuoles is probably Baker's formol-osmium technique. With this method the Golgi element appears pale and more 'vacuolated' than with any of the other methods, but the dense lipid-containing substance is not so heavily osmified.

The Golgi element requires a longer period for osmification in *Rutilus* and *Cyprinus* than in *Gobio*, but it is difficult to see any connexion between this and the nature of the food.

Two silver impregnation methods (Da Fano and Aoyama) were tried but with little success. A faint impregnation of the dense lipid-containing substance occurred in one case only (intestine of *Gobio*) and that by Aoyama's method. Baker experienced a similar difficulty with material from the newt. This surprising result cannot be explained.

If the absorptive epithelium be examined in the active state a noticeably different picture is obtained. If material from a fish which has recently fed on food rich in fats be examined, fat globules will be so abundant in some cells as to mask the Golgi element completely; nevertheless others, which have not absorbed much fat, still show little of the Golgi element. In osmicated material the dense lipid-containing substance appears greatly diminished and no vacuoles of any shape can be seen; nevertheless, in intra-vitam neutral-red preparations vacuoles show up clearly and are seen to be nearer to the free border than is the case in the resting cell. It would thus appear that the vacuoles are normally confined or held in place by the dense lipid-containing substance which disintegrates almost completely during the process of fat absorption. Thus the Golgi element, as well as the mitochondria, is apparently involved in this process.

B. The Goblet Cells

Much of the extensive literature on goblet cells has already been reviewed by Oppel (1896) and by Macklin and Macklin (1932), but there are still some discrepancies in the accounts even of such well-known structures as goblet cells. For example, it is not known whether goblet cells are developed from undifferentiated columnar cells, from modified absorptive cells, or whether they are formed *sui generis*. Again it is uncertain whether goblet cells discharge their contents once and for all, or whether this is a continuous process.

The mucous contents of the cells have been stained by the following methods: Mayer's mucicarmine; Lillie's toluidine blue (Cowdry, 1943); toluidine blue of Hempelmann (1940); periodic acid followed by Schiff's reagent (McManus, 1947), while the zymogen granules were stained either by Bensley's neutral gentian or Bowie's ethyl-violet-Biebrich-scarlet (Cowdry, 1943).

The stoma of the goblet cell is noticeably widened when mucus is being discharged. According to Macklin and Macklin (1932) the luminal end of the goblet cell is thought to be encircled by the intercellular band ('terminal bars') and to be covered by a 'top-plate' which is ruptured to form a stoma when the contents are being discharged. The present observations do not

agree with this. By careful focusing up and down on vertical sections the free border may be seen to be continuous above and below the stoma; thus the stoma is a permanent opening which widens or narrows according to whether mucus is being discharged or not; evidence that the free border is elastic has already been given, p. 327. The stoma of the goblet cell may therefore be regarded as possibly of a similar nature to a large canal of the canal layer of the absorptive cells, or to the passage between two adjacent absorptive cells.

The contents of the goblet are produced in the form of granules which stream out as a shapeless mass. According to Duthie (1933) fixation with Regaud followed by staining with Bensley-Cowdry's method for mitochondria gives a red colour to premucin (mucigen) and a greenish-blue colour to the formed mucus. Applying this technique to the cyprinid gut, goblet cells showing either premucin or mucus may be found scattered haphazardly throughout the folds. Experimental evidence, which will be discussed in fuller detail in a subsequent paper, has been obtained which suggests that the cells of the intestinal epithelium, both goblet and absorptive cells, are regenerated at the bases of the crypts and that they migrate along the sides of the folds to the crests before becoming effete. If this view is correct it is unlikely that the goblet cell discharges its contents once and for all since all stages of mucus formation may be found anywhere on the folds.

The goblet cells of the entire intestine produce zymogen granules. These granules stain more intensely in *Gobio* than they do in *Rutilus* or *Cyprinus*, suggesting that zymogen has a higher concentration in the carnivorous species. Differentially, the highest concentration of zymogen occurs in the rectum and the third limb of the intestine, the stain in the intestinal swelling being quite pale, thus suggesting that protein digestion by enteric proteinases is largely carried out in the posterior segments of the intestine. A further differential effect may be noted, namely, that the granules in the cells at the apices of the folds do not usually stain so intensely as those at the sides but take only a very pale colour with zymogen stains, suggesting that the goblet cell loses some of its potentiality to produce zymogen as it becomes senile while still retaining its capacity to produce mucigen.

The mitochondria of the goblet cell (Pl. I, fig. 1) lie in its basal portion. The Golgi element (Pl. I, fig. 1; Pl. II, fig. 9) is better developed than that of the absorptive cell, while its vacuoles are very variable in size and some of them are far from spherical.

Certain modifications of the goblet cell which appear to give rise to a 'pear-shaped' cell (Pl. I, fig. 1, P.C.R., P.C.G.) will be described in a subsequent communication.

C. The Granular Cells

So extensive is the literature dealing with the granulocytes that occur in various vertebrate classes and so controversial and conflicting are the views of the various authors concerning their appearance, nature, and function

(cf. Michels, 1923; Bolton, 1933), that it is not easy to discuss them briefly. However, two important conclusions can be reached from a survey of this literature, namely, that the cells are present in some species but not in others (Michels) and that, when present, they may be either basiphilic (mastcells or basiphils) or acidophilic (acidophils), while some authors (Michels, 1923; Bolton, 1933; Duthie, 1939) have described hybrid cells showing both basiphilic and acidophilic granules. Bolton claimed that the staining reaction may differ as a result of impurities or variations in the constituents of different batches of dye, while Duthie advises the use of several stains, a single stain being unreliable.

In view of this unsatisfactory state of affairs a revision of previous work is needed and much research, both cytological and experimental, is required before we may fully understand the nature of these cells, and it is not intended, in the present limited study, to devote undue length to this wide problem. An endeavour has been made, nevertheless, to make some contribution to our knowledge of the structure of the granular cells and their function as they occur in gut of fishes. The material studied, in addition to the three cyprinids with which we are especially concerned, includes *Scarus sordidus*, *Mulloidies auriflamma*, and *Atherina forskali* from the Red Sea, *Crenilabrus melops* and *Trigla hirundo* from Plymouth, and *Salmo trutta* from various localities in England. In several cases sections from different species were passed simultaneously through the stains, hence excluding the possibility that differences might be due to impurities or differences in the dye.

The following techniques were employed and the results obtained are tabulated in Table 1:

1. Fixation in Bouin's fluid, stained in haematoxylin and eosin. This was the routine method applied to the Red Sea types in earlier work (Al-Hussaini, 1945, 1946, 1947).
2. Fixation in Zenker-formol, stained with Giemsa (as employed by Duthie).
3. Fixation in Zenker-formol, or Helly's fluid, stained with neutral-gentian of Bensley (as employed by Bolton).
4. Fixation in Helly's fluid, stained with ethyl-violet-Biebrich-scarlet (also employed by Bolton).
5. Fixation in absolute alcohol, stained with thionin (saturated solution in 80 per cent. alcohol) (as used by Michels).
6. Mitochondrial techniques—either Bensley-Cowdry's or Kull's method. So far as is known these techniques have not been previously employed in the study of granular cells.
7. Demonstration of glycogen by Best's carmine method.

A few other methods were applied to the granulocytes of the three cyprinids only; these will be referred to below.

TABLE 1. Reaction of Granular Cells to Certain Fixatives and Stains in Some Teleostean Species

Fish	Techniques as in text						
	1	2	3	4	5	6	7
<i>Rutilus rutilus</i>	Poor; cytoplasm pale pink.	Fair; granules reddish.	Very pale blue almost negative.	Reddish granules.	Poor; few cells show blue granules.	Good; granules red.	Bright red granules.
<i>Gobio gobio</i>	(Granular cells absent)						
<i>Cyprinus carpio</i>	As in <i>Rutilus</i> .	Very few cells show red granules, some bluish.	Pale blue granules.	As in <i>Rutilus</i> .	Few cells show bluish granules.	As in <i>Rutilus</i> .	As in <i>Rutilus</i> .
<i>Scarus sordidus</i>	Very good; red granules.	Very good; red granules.	Good; few cells show blue granules.	Some cells show red, others purple granules.	Negative.	"	Negative.
<i>Mulloides auri-flamma</i>	"	"	"	"	"	"	"
<i>Atherina forskali</i>	Very good; bright red granules.	"	"	"	"	"	"
<i>Crenilabrus melops</i>	As in <i>Rutilus</i> .	"	"	Good, red granules.	As in <i>Cyprinus</i> .	"	"
<i>Trigla hirundo</i>	Very good; bluish-black granules.	Very good; blue granules.	Blue granules.	Purple granules.	Very few cells show pale granules.	Good; blue granules.	"
<i>Salmo trutta</i>	As in <i>Rutilus</i> .	"	Intense blue	"	Pinkish granules.	Good; some cells show blue, others red, granules.	"

It will be seen from the table that the granular cells of the fish gut may be grouped into two main categories: (1) Those which take a blue colour with Giemsa, or the basic component of the mitochondrial stains. These are basiphils and occur only in *Trigla hirundo* and *Salmo trutta*. (2) Those which take a red colour with Giemsa or the acid component of the mitochondrial stains. These are acidophils and are found in all the remaining species where granulocytes occur.

The fact that eosin does not always stain the granules (in *Trigla* they stain bluish-black with haematoxylin-eosin) supports the suggestion made in an earlier paper (Al-Hussaini, 1947) that the granulocytes are not true eosinophils. Jordan and Speidel (1924a) were evidently of this opinion since they describe granulocytes in various teleosts as pseudo-eosinophils. On the other hand, Gulland (1898) described the granular cells in the alimentary canal of the Atlantic salmon as eosinophils, as did Greene (1912) those in the king salmon. More recently Bolton (1933) has called the granulocytes of the sock-eye salmon and some other members of the Salmonidae as basiphilic, thus agreeing with the present observations on *Salmo trutta*.

In agreement with the results shown in Table 1, Duthie (1939) found that the basiphilic granules of *Trigla* were not well fixed by alcohol alone (column

5), though when mercuric chloride is added (e.g. Zenker-formol, column 2) perfect fixation is obtained. On the other hand, Bouin's fluid, though without mercuric chloride, fixed the granular cells of *Trigla hirundo* well (column 1). Bouin's fluid also proved satisfactory for the acidophils of *Scarus*, *Mulloides*, and *Atherina*. Preparations 6 years old, mounted in balsam, are still keeping very well (Pl. II, fig. 10). In contrast to this, the granulocytes of *Rutilus* (Pl. II, fig. 11), *Cyprinus*, *Crenilabrus*, and *Salmo* are poorly preserved by Bouin's fluid. Michels (1923) did not find Helly's fixative so satisfactory for preserving the granules in the 'mast cells' of *Leuciscus* sp. (allied to the roach) as those in *Cyprinus carpio*. He accordingly abandoned aqueous fixatives in the belief that the granules are rapidly dissolved by them and used absolute alcohol, but, as shown in Table 1, column 5, this would appear to be the least reliable of all the fixatives used in the present work. His work is criticized by Jordan (1926) who claimed that the so-called 'mast cells' of *Leuciscus* sp. must be acidophils and thought that the metachromatic staining of the granules by thionin as described by Michels must have been due to impurities in the dye. Duthie (1939), however, repeated Michels's work on the common carp and found that the granulocytes in the intestine are identical with those of the blood and other tissues, and that they all give basiphilic reactions. As may be seen from Table 1, the results of the present investigation show that only a few granules are preserved by alcohol and they stain orthochromatically (i.e. blue) in *Rutilus*, *Cyprinus*, and *Crenilabrus*. The reaction is especially strong in *Rutilus* and, although the granules stain less intensely in *Cyprinus* and *Crenilabrus*, they are far from being reddish. These results, therefore, agree with those of Jordan rather than with Michels or Duthie.

A further complication is introduced by the view put forward by Duthie and several other authors (cf. Duthie, 1939) that orthochromatic (or basiphilic) staining precedes metachromatic (or acidophilic) during the life of a cell. Jordan and Speidel (1924a, b) and Jordan (1926) also claimed that a progressive increase in the acidophilic quality of the granules is part of a ripening process. On this basis Duthie considers that the maturation of the coarse granulocytes is rapid in the Labridae but slow in the Triglidae. It will be seen that the Salmonidae resemble the Triglidae in this respect. Duthie, however, is inclined 'not to ascribe much importance to a colour change in the granules since it is probably influenced by physico-chemical factors in the surrounding tissues'. The present investigation can make no contribution to this aspect of the problem, but it is worth noting that in *Mulloides auri-flamma* and *Salmo trutta* some of the granular cells exhibit both types of granules, acidophilic and basiphilic, while others behave differently according to whether they are outside or inside the intestinal epithelium. In *Trigla hirundo* the granular cells invading the epithelium remain entirely basiphilic even though they may migrate right up to the free border, but in *Atherina* and *Scarus* they remain acidophilic under similar conditions.

It is obviously impossible to draw general conclusions from the above data, and the picture is still further complicated by the fact that the number of

granules which are stainable by one technique may be quite different from that seen with a different one, even in one and the same fish. For example, with mitochondrial techniques (No. 6) numerous granules are stainable in the granulocytes of *Cyprinus*, but only a small number are visible with techniques 2, 3, or 4. This suggests that even in one species the granules may be of differing chemical composition and hence may have several functions to perform.

Drzewina (1911) noted that the granulocytes disappeared from the blood and alimentary tract of certain Labridae during fasting but returned and were maintained in these sites during feeding. I have also noted a similar behaviour in granulocyte activity in *Mulloides auriflamma* (Al-Hussaini, 1946), but in *Rutilus* and *Cyprinus* their distribution is not connected with the presence or absence of food in the gut and most other authors make no mention of any such relationship. In order to try to discover more precisely the nature of the stimulus which evokes the migration of the granular cells into the intestinal epithelium of *Mulloides*, four specimens of about equal weight were kept without food for 3 days, and then on the fourth were given each an equal quantity of a semifluid diet of approximately the same consistency, but of different food content. The food was given through the mouth with a pipette and great care was taken to apply as nearly as possible the same pressure in each case. The first fish was given a suspension of casein, the second one of starch the third an emulsion of castor oil, and the fourth an inert substance (magnesium trisilicate in liquid paraffin). They were killed 4 hours later and sections of the intestine were prepared. Granular cells were present in equal profusion in the stomach wall of all four fish; hence since each kind of food was equally effective in producing a response as also was the inert magnesium trisilicate and liquid paraffin, the stimulus would thus appear to be due rather to the mere physical presence of material in the lumen of the gut than to its chemical nature or nutritional role. Perhaps the increased peristalsis occasioned thereby may play a part.

Some evidence of a wandering tendency in the granulocytes was observed in all the species examined. In *Rutilus* and *Cyprinus* the cells have never been observed to migrate farther towards the lumen than the basal third of the absorptive cells, whereas in the other fishes examined they migrate as far as the free border and in the majority they are ruptured as though discharging their granules. They are, of course, entirely absent from *Gobio*. *Rutilus* shows an interesting condition which appears to be so far unrecorded, in that granular cells are encountered in the pulp cavities of the pharyngeal teeth where they may be seen within the capillaries, alongside erythrocytes, as well as within the surrounding connective tissue. They were, however, never seen in the large blood-vessels, neither could they be demonstrated in blood films prepared by the usual routine methods. This, however, may well be due, as Drury (1915) suggested in a similar instance, to the instability of the granules.

The granular cells of *Rutilus* and *Cyprinus* show a positive reaction to the histochemical test for alkaline phosphatase (Pl. III, fig. 13), *Rutilus* showing

a stronger reaction than *Cyprinus*. They also store glycogen (cf. Table 1, column 7).

Summaries of the ideas of various authors concerning the possible functions of the granular cells have been given by Bolton (1933) and by Duthie (1939), while I have also made a brief reference to the subject in an earlier paper (Al-Hussaini, 1946). They are usually credited with a secretory function of some sort, either exocrine or endocrine, and may possibly be concerned with lipase activity and/or the elaboration of zymogen. Certainly the granular cells of *Salmo* and *Trigla* give a positive colour test for zymogen as, in a less degree, do those of *Scarus*, *Mulloidés*, *Atherina*, *Cremilabrus*, *Rutilus*, and *Cyprinus*. Jordan and Speidel (1924b), on the other hand, believe that the 'intestinal eosinophils' (granular cells) may relate to the general immunity of the mucosa to the bacterial content of the gut by the elaboration of anti-toxins. As suggested above, it is highly probable that the granular cells perform different functions in different species or even within any one species. Their wandering propensities suggest that they are capable of responding to diffuse stimuli operating in a fluid medium and occasioned by the several special needs of the organism. Their capacity to store glycogen in *Rutilus* and *Cyprinus* would partly meet the needs of fish whose food is rich in carbohydrates and may account for their absence in the carnivorous *Gobio* belonging to the same family, but this is obviously not the whole story and their activities in the numerous other fish in which they occur still remain to be discovered.

Discussion of Cytology

The free border of the intestinal cells in the lower chordates bears cilia which assist in the movement of food particles through the alimentary canal and is associated, in general, with ciliary feeding mechanisms. The evolution of the jaws produced a new mechanism for obtaining food and enabled the resulting gnathostomes to secure bulkier food; hence ciliated epithelium as a gut lining lost much of its original purpose and the result has been the evolution of a continuous free border pierced by fine canals as described by Baker for tetrapods and here for many teleosts. *Pterois volitans* and *Scarus sordidus* present free borders of unusual appearance, neither ciliated nor with typical canals, but show a condition that can be interpreted as intermediate between these extremes. It is highly desirable that an extensive survey of the free borders of the intestinal epithelium of lower vertebrates should be made to test this idea, and possibly to link the aberrant types together into an evolutionary scheme.

The fact that the free border is not of constant thickness throughout various parts of the intestine of the same fish suggests that this thickness can be, and normally is, altered from time to time—a feature probably concerned primarily with the absorption of digested food into the cells. Baker, indeed, goes farther and suggests that the opening and closing of the pores of the canals 'may be one of the reasons for the movements of the villi and an explanation of their musculature'.

The goblet cells produce both mucus and zymogen simultaneously and these products appear to pass together through the permanently open stoma into the lumen of the gut where they imbibe water, swell, and pass into solution. This permanent stomatal opening may well correspond to a specially enlarged canal of the free border of the absorptive cell and its narrowing and widening are probably influenced by movement of the intestinal folds in the same way.

Excellent comprehensive reviews on the roles of mitochondria and the Golgi element in animal tissues in general are given by such authors as Cowdry (1924), Bowen (1929), Bourne (1942), Hibbard (1945), and Worley (1946), and it is not proposed to extend that discussion beyond saying that the evidence obtained during the present investigation gives strong support to the view that mitochondria are intimately concerned with the process of fat absorption. The Golgi element also disintegrates during fat absorption (a finding supported by Cramer and Ludford, 1925, and by Liu, 1930), but this is certainly not the only role played by this structure. There is considerable agreement amongst previous workers, e.g. Brambell (1925), Bowen (1926, 1929), Ma (1928), and Worley (1944), that the Golgi element is concerned with secretion. Nassonow (1923) declares that spheres of mucus originate within it. Such evidence as the present investigation can supply is twofold: first the indirect evidence that the Golgi element is larger and better developed in the goblet cell than in the absorptive cell and secondly that the part of the cell where this structure lies may show a positive reaction to alkaline phosphatase (cf. p. 339).

The occurrence and peculiar staining reactions of the granular cells so characteristic of the gut of teleosts, and which, in spite of their diverse basiphilic and acidophilic properties, are probably generically comparable throughout, have already been discussed at length (pp. 331-6) and it is not proposed to re-enter that discussion here.

II. THE PHYSIOLOGY OF THE INTESTINAL EPITHELIUM

A. *Histochemical Studies*

1. *Hydrochloric Acid*

The presence of free acid was first demonstrated in the mammalian stomach by Claude Bernard and, although his procedure, involving the injection of potassium ferrocyanide into the animal, has been variously modified, all subsequent techniques employ the same basic principle (cf. Lison, 1936). The modification of FitzGerald (1910) is the one employed here. No precipitate of Prussian blue was found in the gut of any of the fishes examined and hence it may safely be inferred that free HCl did not occur. The fact that the injected ferrocyanide solution had indeed passed into the mucosa was confirmed when sections of the intestine were stained for glycogen by the Bauer-Feulgen technique, the chromic acid producing a copious blue precipitate in the epithelium and the tissue immediately subjacent to it. No other worker has so far succeeded in finding HCl in

the cyprinid gut; thus one may safely conclude that the stomachless cyprinid has lost one of the principal attributes of the stomach and has not evolved acid digestion in any other organ.

2. Lipase

Gomori (1945) has recently succeeded in demonstrating the intracellular sites of lipase activity by histochemical methods. He used as a substrate either palmitic or stearic esters (known respectively as 'Tween 40' and 'Tween 60', manufactured by the Atlas Powder Co. of America). The substrate used in the present study was a gift from Dr. Gomori himself and is a 'product No. 81 of the Ornyx Oil & Chem. Co., Jersey City, U.S.A.'. Dr. Gomori, in his letter, described it as 'a stearic acid ester of a glycol of high molecular weight which is an excellent substrate for the histochemical demonstration of lipase'. For full details of the method reference should be made to Gomori's paper, but in essence it consists of first splitting the substrate by means of the lipase in the tissues, then precipitating the acid radicle as the calcium salt. This is then converted into the corresponding lead salt which, by treatment with ammonium sulphide, is finally transformed into the dark brown or black lead sulphide. Thus, on counterstaining with haematoxylin, dehydrating and clearing in dichloroethylene (*not xylene*) the sites of the original lipase activity may be clearly seen as shapeless blotches, dark brown or black in colour, of no constant size or shape which, under the oil-immersion, are seen to have a granular texture, the granules sometimes fusing into larger masses. Treatment of the slide before incubation with Lugol's iodine solution for one minute completely inhibits the reaction and thus provides a convenient method of control.

Positive results were obtained in all three cyprinids in the mucosal epithelium and in the pancreatic alveoli surrounding the intestine, while some lead sulphide was also deposited in the lumen of the intestine. The absorptive cells show the highest concentration of the enzyme (Pl. III, fig. 16), but a few goblet cells also show very meagre deposits of lead sulphide which in no way correspond to the zymogen granules. It is possible that the enzyme may have got there by diffusion rather than by secretion, especially since it also occurs free in the intestine. The important conclusion to be drawn from this result is that the absorptive cells are secretory as well as absorptive.

The highest concentration of the enzyme occurs in *Gobio*, where the mucosal folds are all loaded with the deposit. However, it decreases in intensity in a cranio-caudal direction. A similar general picture was obtained for *Cyprinus* and *Rutilus*, but the lipase activity in the third limb of the intestine and the rectum is very weak and it may even be absent entirely from these portions of the gut in *Rutilus*.

During the biochemical estimation of enzyme activity (p. 345) extracts from the wall of the buccopharynx were found to give a positive reaction for lipolytic enzymes. The histochemical test was accordingly repeated several times and on several individuals but was entirely negative except in one

instance, namely, a piece of material from the pharynx of *Cyprinus*, deposits of lead sulphide occurring in the connective tissue amongst the adipose cells scattered in the wall. It is well known that fat-storing tissues contain lipase (Maclean, 1943), nevertheless Gomori (1946) failed to get positive results with his test on the adipose tissues of rhesus monkey, dog, and guinea-pig, although he succeeded with similar tissue from the rat and rabbit. Gomori also found that the stratified epithelium of the oesophagus of several species of mammals contains lipase; the stratified epithelium of cyprinids, however, proved entirely negative. The possible causes of this discrepancy between histochemical and biochemical results is further discussed on p. 345.

3. Phosphatases

It is to Gomori again that we owe satisfactory methods for the histochemical demonstration of both alkaline phosphatase (Gomori, 1939) and acid phosphatase (Gomori, 1941b). These methods have been used in the present work and the reader is referred to Gomori's original papers for technical details. Reference should also be made to Danielli's critical review (1946). All experiments were run with appropriate controls, namely, control of the method by running sections of rat kidney and fish kidney through the solutions simultaneously with those of the gut and control of any possible pre-existing calcium in the tissues by passing identical slides through the various stages but *omitting* the incubation stage. Any calcium present will react with the lead nitrate and produce a deposit, whereas phosphatase will not do so unless incubated with a substrate.

All tests for acid phosphatase were negative. Alkaline phosphatase was most abundant in the free border of the absorptive cells of all species (Pl. III, fig. 14). The superficial layer appears jet black, the canal and granular layers paler, the 'clear zone' still paler, while the rest of the cytoplasm showed no appreciable coloration. In *Rutilus* and *Gobio* the whole intestine from swelling to rectum exhibits this activity, although with considerably decreasing intensity in the more caudal regions. *Cyprinus* gives a somewhat similar picture except that the decrease in intensity is even more striking, the rectum showing hardly any phosphatase activity at all. Phosphatase activity is also seen in the subepithelial connective tissue; the fibres take a light brown colour while, in *Cyprinus*, the collagen fibres lying just internal to the circular muscle appear brownish-black. Blood corpuscles react positively and the granular cells of *Rutilus* and *Cyprinus* also show some activity (Pl. III, fig. 13). In *Gobio* the stratum compactum contains alkaline phosphatase (Pl. III, fig. 14).

The Golgi zone showed a positive reaction to the tests in a few absorptive cells only (Pl. III, fig. 15), so that it cannot be considered a constant feature in the cyprinid gut.

The walls of the bucco-pharynx contain considerable quantities of the enzyme. Its activity is greatest in the basal layer of the stratified epithelium and decreases gradually towards the surface (Pl. IV, fig. 17) (cf. also Al-Hussaini, 1948).

The results described above confirm the conclusions of Gomori (1941a), Kabat and Furth (1941), Bourne (1943), and others concerning the concentration of the enzyme in the free border of the absorptive cells, but they are at variance with the results of Emmel (1945) and Deane and Dempsey (1945) who consider that the location of the enzyme in the Golgi zone is a constant feature of intestinal epithelia.

4. *Absorption*

In order to demonstrate any possible differences in the absorptive capacity of different regions of the intestine or in the intestines of the different fish, it was felt that the absorption of fat would be likely to yield the most conclusive results as it is a process that has been much studied in mammals though little in fish. Herwerden (1908) during the course of his study of gastric digestion studied the absorption of fat from the stomach of several fishes, as did Greene (1913) in the king salmon and Dawes (1930) in the plaice.

The technique adopted here has been to fast the fish for some days and then administer, usually through the mouth, a small quantity of castor oil. The fasting period needs to be lengthy (2 weeks for *Gobio*, 3 for *Rutilus* and *Cyprinus*) since the fish intestine does not become empty so quickly as that of mammals. Food usually leaves the intestinal swelling fairly soon, but it takes much longer to clear the gut entirely (this agrees with other workers on stomachless fishes, cf. Barrington, 1942). It is important to change the water in which the fish live once or twice daily during the fasting period to prevent the fish from eating their own faeces.

According to Cameron (1945) castor oil is the glyceride of ricinoleic acid (a hydroxy derivative of oleic acid). It is not proposed to enter into a detailed discussion concerning the various implications and theories of fat absorption, but rather to give a straightforward account of the phenomenon as it could be seen in the three cyprinid fishes investigated.

Only two simple techniques were used to demonstrate fats, namely, (1) impregnation by means of osmium tetroxide by either Schridde's or Kolatchev's method (it is important to realize that OsO_4 has only a morphological, not a histochemical value, cf. Lison, 1936), (2) a modification of Ciaccio's method for 'lipoids' (= lipines, cf. Lison). The modification consists of staining by Sudan III in 90 parts 80 per cent. alcohol and 10 parts acetone instead of in 95 parts alcohol and 5 parts acetone. This was found to be more satisfactory for fish material. Although, according to Lison, Kaufman and Lehmann have criticized this method they accept it for non-saturated fatty acids; ricinoleic acid is such an acid and the method is therefore suitable for its detection.

With the osmium tetroxide techniques the absorptive cell in the fasting condition displays a dense granular cytoplasm (due mainly to mitochondria which are sometimes impregnated with OsO_4) and a Golgi element. A similar picture is obtained with Ciaccio's method except that the Golgi element is masked.

During active digestion of a normal diet the absorptive cells appear vacuolated when studied by methods other than those designed to preserve and stain fat (Pl. IV, fig. 18). Dawes (1929) found that the 'columnar cell' during the resting phase shows a deeply stained mass which becomes smaller during the active phase. He believes that the appearance of vacuoles is directly related to the presence of food in the lumen. Rogick (1931) thinks that the vacuoles represent a phase in the formation of goblet cells; McVay and Kaan (1940), however, exclude the possibility but offer no alternative explanation of the phenomenon. On the basis of experience gained during the present investigation a more probable explanation would seem to be that the 'vacuoles' simply represent the loci occupied by fat globules, the fat having been dissolved out by the alcohols and xylene used in preparing the sections.

It is convenient to describe the condition of the epithelium during fat absorption by means of seven protocols each describing a different experimental procedure and its result.

Protocol 1. A fasting roach 14.0 cm. long was given about 1 c.c. castor oil through the mouth and killed 19 hours afterwards. Transverse sections of various parts of the intestine were prepared and treated by Kolatchev's and Schridde's osmium techniques.

Fat globules of very variable size are found in the absorptive cells (Pl. IV, fig. 19); some may attain a diameter of 8μ while others measure less than 1μ . The free border is quite clear of globules and the clear zone is almost free from them. Next to the clear zone there are usually a few globules of small diameter followed immediately by globules of the largest size; indeed the large globules are confined to the luminal half of the cell. Here and there between the clear zone and the nucleus minute globules also are encountered, but the majority of them are found in the basal half of the cell.

The cells from the various parts of the intestine have not absorbed equal quantities of fat. In the intestinal swelling the greatest absorption has occurred on the crests of the mucosal folds, although the cells as far as half-way down the sides of the folds may contain fat. In the second limb of the intestine almost every cell is loaded with fat, while in the third limb, although almost every cell contains fat globules, their concentration is not so dense as in the second limb. In the rectal epithelium practically every cell is clear of fat.

The tunica propria also contains fat globules, but they are not as abundant as in the absorptive cells. The globules here are usually small, but sometimes shapeless masses, apparently formed from fused globules, may be seen in the meshes of the connective tissue. These masses are not usually so heavily impregnated by osmium as the globules themselves. Fat globules are extremely rare within the submucosa.

Protocol 2. A fasting roach 18.4 cm. long was given about 1 c.c. of castor oil by mouth and killed $43\frac{1}{2}$ hours later. Transverse sections of various parts

of the intestine were prepared and the fat was stained by Schridde's and Ciaccio's methods.

More cells were loaded with fat in the intestinal swelling than in protocol 1; even those at the bases of the mucosal folds contained fat. In general the fat globules were smaller and none was so large as $8\ \mu$ diameter. The second and third limbs present pictures comparable with those obtained before (Pl. IV, fig. 20). This time the rectum has also absorbed some fat, mostly along the crests of the folds. By comparison with the state of affairs in the fish killed only 19 hours after feeding it may be seen that many fat globules have passed inwards towards the nucleus (Pl. IV, cf. fig. 19 with fig. 20).

Small fat globules are still of frequent occurrence in the tunica propria but are not as numerous as formerly. In addition to the fat globules stained red, unstained 'vacuoles' also appear in the sections prepared by Ciaccio's method, but these 'vacuoles' do not appear in the material osmicated by Schridde's method, thereby suggesting that some of the fat at least is altered during the absorptive process.

Protocol 3. A fasting gudgeon 11.0 cm. long was given about 1 c.c. of castor oil by the mouth and killed about 21 hours afterwards. Transverse sections of various parts of the intestine were prepared and the fat stained by Kolatchev's and Ciaccio's methods. With the osmium impregnation (Kolatchev's method) a picture was obtained closely comparable with that described in protocol 1, except that some of the cells on the crests of the mucosal folds of the rectum have absorbed fat. Large masses of fat also occurred in the blood-vessels and lymph spaces in the tunica propria. With Ciaccio's method Sudan positive globules of varying sizes occur in the absorptive cells on the crests of the mucosal folds, being most abundant in the basal portions of the cells.

Protocol 4. A fasting gudgeon 11.4 cm. long was given about 1 c.c. of castor oil and killed 67 hours later. Transverse sections of the intestine were prepared as in protocol 3. Only a small number of fat globules were present. They were recognizable by either method and were present throughout the intestine but occupied only the crests of the mucosal folds.

Protocol 5. A fasting mirror carp 10 cm. long was given about 1 c.c. of castor oil by the mouth and killed 4 hours later. Transverse sections of the various parts of the intestine except the rectum were prepared by Kolatchev's technique. The absorptive cells showed considerable accumulations of fat, and, as in protocol 1, the larger globules were found in the luminal halves of the cells. Abundant fat globules were also present in the tunica propria.

Protocol 6. A fasting mirror carp 12.5 cm. long was given about 2 c.c. of castor oil through the anus and killed 21 hours later. Sections of the intestine were prepared as in protocol 3. In the osmicated preparations the resulting picture was similar to that in protocol 1, but by Ciaccio's method globules stained red by the Sudan III occurred in the intestinal cells but not in the connective tissue.

Protocol 7. A fasting mirror carp 15 cm. long was given about 0.2 c.c. of castor oil through the mouth and killed 21 hours later and sections of the gut were prepared as in the previous example. This time only the absorptive cells on the crests of the mucosal folds of the intestinal swelling showed any sign of fat globules.

The following conclusions may be drawn from the above experiments:

1. Fat is mainly absorbed by the second and third limbs of the intestine, the intestinal swelling absorbing less and the rectum very much less; indeed the amount absorbed by the rectum must be very small (protocols 1, 2, 3, 4, and 6). From protocol 7 it might appear that when the quantity of fat is very small it is entirely absorbed by the intestinal swelling, but much more supporting evidence would be needed before one could feel justified in making this generalization.
2. Early in the fat-absorbing process fat globules of widely varying size are found, some reaching considerable dimensions (8μ diam.), while later they become more uniformly small (protocols 1, 2, 3, 4, 5, and 6).
3. The fat passes from the absorptive cells into the tunica propria, either into blood-vessels or lymph spaces. The submucosa contains little or no fat (protocols 1, 2, 3, and 5).
4. Some modification occurs to at least some of the fat during the absorptive process since the pictures obtained by means of the osmium techniques show a greater concentration of globules than those obtained by the more selective Sudan III methods (protocols 2 and 6).

5. *Glycogen*

For the demonstration of glycogen three methods were employed: (1) Best's alkaline carmine technique following alcohol-formol fixation. (For a discussion on the reliability of this method see Davies and Francis, 1941.) (2) Bauer-Feulgen's method, also after alcohol-formol fixation. (3) The method originally designed by McManus (1947) as a mucus stain but, as Dr. J. F. A. McManus has said in a personal communication to the author, the method seems to show carbohydrates and their compounds such as glycogen, mucins, &c. In each case the saliva test was used to differentiate between glycogen and galactogen, the former being hydrolysed thereby while the latter is not (Lison, 1936).

Glycogen was found only in the granular cells of *Rutilus* and *Cyprinus*, *Gobio* giving only negative results (cf. also Table 1).

B. *Biochemical Studies*

A study of the literature reveals that the estimation of enzymes in the intestine of the carp has yielded somewhat conflicting results. This seems to be due to the fact that the investigators have failed to realize, either wholly or partially, that the gall-bladder and intestine are intimately invested by fatty tissue containing pancreatic alveoli, and hence special precautions are needed

without the preliminary precaution of fixing the pancreatic tissue and of the intestine and pancreas together were also tested for comparison.

TABLE 3. *Enzymatic Activity of the Alimentary Canal and Bile of R. rutilus, G. gobio, and Cyprinus carpio, symbolically expressed*

<i>Part examined</i>	<i>Enzyme</i>	<i>G. gobio</i>	<i>R. rutilus</i>	<i>Cyprinus carpio</i>
Buccal and pharyngeal walls	A	—	+	+
	P	—	—	—
	L	+	++	++
Intestinal swelling	A	+++	+++	++++
	P	++	++	+
	L	+++	+++	+++
Second limb of intestine	A	+	+++	++
	P	++	++	++
	L	++	++	++
Third limb of intestine	A	+	+	+
	P	++++	+++	+++
	L	—	Very slight	Slight
Bile	A	—	Very slight	Slight
	P	—	—	—
	L	—	—	—

A — Amylase. P — Proteinase. L — Lipase.

The results, given in Table 3, are expressed by symbols rather than numerically since it was felt that numerical results based on such small quantities (from 1.0 to 2.0 c.c. of extract) of inevitably impure material might well result in the appearance of tempting comparisons and relationships altogether unjustified by the circumstances. The results obtained from the preparations in which no preliminary fixation of pancreatic tissue was done showed definitely stronger reactions, while from those in which the extract was made from intestine and pancreas together gave stronger reactions still.

From the results we may reach the following conclusions:

1. The results of biochemical tests for proteinases correspond with those of the histochemical tests for zymogen granules.
2. The biochemical estimation of lipase activity also corresponds pretty closely with the histochemical tests, with the exception of tissue from the bucco-pharynx, which gave positive results with the biochemical method but failed to do so (with the exception of one individual) with the histochemical one. Presumably this is due to qualitative rather than quantitative causes since the third limb of the intestine contains relatively very much smaller quantities of the enzyme, but this was easily detectable by both methods. It is of interest to note in this connexion that Maclean (1943) got biochemical evidence of strong lipase activity in the mammalian spleen, but Gomori (1946) failed to demonstrate it by his histochemical test. Vonk (1937) calls attention to the fact that the specificity of this group of ferments (lipases) is not very

pronounced, while Frazer (1946) says that 'lipase may be one enzyme . . . or it may consist of a group of esterases'. Further comparative work as between biochemical and histochemical results should yield data of considerable interest.

3. Both lipolytic and amylolytic enzymes show a maximum concentration in the intestinal swelling and decrease gradually towards the caudal end of the gut.

4. Proteolytic enzymes, on the other hand, are more concentrated in the third limb of the intestine than in the more cranial segments. Assuming the histochemical test to be a safe guide, the rectum is at least equal to the third limb in this respect (biochemical assay could not be applied to the rectum, *vide supra*).

5. Bile, apart from a very slight amylolytic activity in the herbivorous *Cyprinus* and omnivorous *Rutilus*, is practically devoid of enzymes.

6. The mucous membrane of the bucco-pharynx shows amylolytic activity in *Rutilus* and *Cyprinus* which, though slight, is stronger than that of bile. In all three forms it shows lipolytic activity. This is probably concerned with fat storage and with the utilization of fat stored in the submucosa rather than with digestive processes (cf. also pp. 338 and 339).

7. The concentration of carbohydrases is in direct relation to the relative amounts of carbohydrates in the food of the fish, being highest in *Cyprinus* and least in *Gobio*, while the protease activity is highest in *Gobio* and lowest in *Cyprinus*, being thus directly correlated with the amount of animal food the fish eats; this agrees with comparable results obtained by earlier workers. For example, both Kenyon (1925) and Vonk (1927) correlated the strength of the enzymes with the nature of the diet; Kenyon found that the hepatopancreas and the intestine of the common carp both secrete a much more powerful amylase than do those of the carnivorous pickerel, while Vonk found the strength of the proteinases in the common carp to be six to eight times weaker than those of the pike. Further, Ishida (1936), who investigated four different species of stomachless fish, found amygdalase and salicinase in herbivorous fish only, never in carnivorous. Finally, Grossman *et al.* (1944) have shown that by substituting glucose for corn starch in a balanced diet fed to rats an increase in the amylase content of the pancreas results.

Discussion of Physiology

Kenyon (1925), Vonk (1927), and Yonge (1931) have shown that digestion in fishes is essentially the same as in other vertebrates, but Babkin and Bowie (1928) distinguish between the acid-alkaline type in which a stomach is present and free HCl is accordingly secreted, and the exclusively alkaline digestion of the stomachless fishes. With the loss of a stomach, pepsin digestion is also lost, although Krukenberg (1877-8, quoted from Biedermann, 1911) claimed to have found that the mucous membrane of '*Cyprinus tinca*', in January, secretes a strong pepsin. Digestion in the three cyprinids which form the basis of the present study is exclusively alkaline.

The localization of enzymes in cyprinids has been the subject of much controversy, due mainly to inaccurate definition of the intestinal segments. Thus Luckhau (1878) found that the 'anterior intestine' secretes both amylase and proteinase and Knauthe (1898) demonstrated a strong proteinase in this part of the gut. Yung (1915) found that the entire intestine produces a weak proteinase as also does the hepato-pancreas. He claimed further that if these two weak enzymes are mixed together their action is considerably strengthened. Kenyon (1925) maintains that the intestinal secretions of the carp show only very weak tryptic digestion, and he thinks that, in such fishes with a diffuse pancreas, the trace of tryptic digestion found may be due to small ramifications of pancreatic tissue embedded in the intestinal wall. He identified the intestinal proteinase as consisting mainly of erepsin—as is normal in other vertebrates. Vonk (1937) believes that the intestinal mucosa of the carp can furnish an erepsin as well as an enterokinase, but that the amylase present comes from the diffuse pancreas. Finally Carrié (1937) asserts that the intestine of the tench produces no enzymic secretion at all, a finding unsupported by any other workers on this or related species.

Although no attempt has been made in the present study to identify individual enzymes in the three principal categories, yet it has been clearly shown that not only does the intestinal mucosa secrete enzymes, but that the various regions of the intestine exhibit this property in different degrees. It is suggested that these conclusions merit full consideration for two reasons, namely, the preliminary fixation of the pancreatic tissue precludes the possibility of the mucosal scrapings being contaminated from this source, and secondly the *in vitro* proteolytic and lipolytic biochemical tests are in accord with the histochemical investigation. Unfortunately no comparable histochemical technique for carbohydrates is available; if such could be devised it would fill the gap and make complete confirmation possible.

According to Biedermann (1911), Krukenberg (1878) has shown that the buccal mucosa of the carp secretes a diastase. Neither Knauthe (1898) nor Beauvalet (1933*b*) was able to confirm this. As can be seen from Table 2, the results of the present work lend support to Krukenberg's contention, since a diastase, though weak, is undoubtedly present in the mucosa of the buccopharynx of the mirror carp and roach, though it is absent from the gudgeon.

How far then has the loss of a stomach affected the balance of secretion? We learn from Beauvalet (1933*b*) that, according to his own results (1933*a*) and those of others, when fishes have a stomach the secretions of their intestines do not affect proteins to any appreciable degree, whereas in stomachless fish, this class of foodstuffs is capable of being digested along the entire length of the gut, a conclusion completely in accord with results of the present work. This finding is an important one, for it appears that the loss of a stomach and consequently of peptic digestion has been compensated by the development of a strong tryptic (? ereptic) enzyme by the intestine which augments strong enzymes of a similar character elaborated by the hepato-pancreas. Other cases of enzymic substitution are known to occur in fish,

e.g. Beauvalet (1933a) compared the digestive capacity of the hepato-pancreas of *Ameiurus* and *Micropterus* and found it much higher in the former fish since the enzymes from the hepato-pancreas are augmented in *Micropterus* by those of the pyloric caeca, which structures are absent in *Ameiurus*. Chesley (1934) made comparable studies on other fishes and came to similar conclusions.

Since the stomach is also a receptacle for storing food it follows that a fish with a stomach can take in a greater mass of food at any one time than can a stomachless fish, making it necessary for the latter to feed at shorter intervals. This permits the digestive enzymes to mix more thoroughly with the food and consequently their action is accelerated (cf. Barrington, 1942). Thus the elaboration of tryptic enzymes by the intestine and more frequent feeding resulting in their more efficient use offers a dual compensation for the loss of the stomach.

The function of alkaline phosphatase has attracted a host of workers during the last decade. Evidence that phosphatase may be directly related to glucose absorption was first advanced by Lundsgaard (1933). Indirect evidence that this enzyme is concerned with absorption is offered by the present investigation which has shown that alkaline phosphatase is especially concentrated along the free border of the absorptive cells, that is, in direct contact with the digested food. Its presence in the tunica propria may well be associated with the passage of food materials from the absorptive cells into the body fluids. According to Bourne (1943) it is not known for certain whether the phosphorylation of sugars is carried out in the lumen of the intestine or whether the sugar is first adsorbed on to the surface of the cell, or absorbed into the brush border (canal layer), phosphorylated, and then passed into the interior of the cell.

The relation between alkaline phosphatase and glycogen is also a generally accepted thesis. Loci of glycogen in various tissues have been shown to be also sites of phosphatase activity, as, for example, in the liver (Gomori, 1941a), placenta (Wislocki and Dempsey, 1946), in the hair (Johnson and Bevelander, 1946). In the present work a similar association between phosphatase and glycogen has been observed in the granular cells of *Rutilus* and *Cyprinus*.

The stratified epithelium of the buccopharynx also contains alkaline phosphatase, the highest concentration being in the stratum germinativum. It might be argued, on the grounds of the above hypothesis, that the enzyme might here act in an analogous way to that in the free border of the absorptive cell or the brush border of the kidney tubule, but this time by absorbing glucose from the tunica propria rather than from the lumen. In such epithelia as that lining the buccopharynx the intercellular substance is very scanty, and the intake of glucose for purposes of nutrition may perhaps be effected thus by a chemical process of phosphorylation rather than by the physical process of diffusion (cf. Al-Hussaini, 1948).

For the tremendous literature on fat absorption the reader is referred to Verzá and McDougall (1936), Frazer (1946), and Nielsen (1946). Frazer

reviews two hypotheses of fat absorption: (1) the lipolytic hypothesis advanced by Verzár and McDougall and (2) the partition theory put forward by Frazer himself. About the first he says 'it maintains that fat is completely hydrolysed in the intestinal lumen, that the intestinal cell has an outer pavement membrane, that paraffins are not absorbed, etc.'. Frazer's own hypothesis is based on the partial hydrolysis only but the complete emulsification of fat. Thus he believes that fat globules of less than $0.5\ \mu$ in diameter may be absorbed without being hydrolysed, and that the intestinal absorptive cell has a porous free border (a point amply confirmed by the present work) which would allow the passage of such minute globules through its canals so that paraffins, which are not affected by lipase, can be absorbed. As regards the phosphorylation of fat, the lipolytic theory claims that it is a stage in the resynthesis of fat, while according to the partition hypothesis it is not concerned with resynthesis but occurs at the oil/water interface, producing an essential change in the interfacial structure, the phospholipid being added to the fat particles in the intestinal wall.

So far as the present investigation is concerned one of the main results is the demonstration that fat is first dealt with by the cells on the crests of the mucosal folds and that absorption later spreads down the sides of the folds to their bases. There does not appear to be a direct relationship between the amount of fat absorbed and lipase content (as demonstrated histochemically) of the tissue absorbing it. Thus the lipase activity, as shown by both histochemical and biochemical assay, is highest in the intestinal swelling and lowest in the third limb and rectum, but experimental evidence suggests that the greatest amount of fat is absorbed by the second limb. Evidence has been brought forward to suggest that lipases may vary qualitatively and that lipase may be detectable biochemically, and thus be physiologically significant, yet fail to yield positive results with the histochemical test employed.

The fact that no fat globules have been encountered in the free border might appear to discount the partition theory, since this is essentially based on the passage of fat globules through the canals of the canal layer; on the other hand, it may be that this passage is accomplished very rapidly and hence evades histological demonstration.

A number of observations have been made concerning fat globules in the tunica propria. Dawes (1930) found Sudan positive globules in this tissue in the plaice while Herwerden (1908), working with various elasmobranchs and teleosts, has described the transfer of fat globules from the epithelial cells of the stomach into the lymph spaces accompanying blood-vessels. Barton (1902) working on kelts, and Greene (1913) on the king salmon, found that fat absorbed by the intestinal mucosa passed into the tunica propria. The results of the present work are entirely in agreement with these findings and have shown that fat is transferred from the absorptive cells of the intestine to the lymph and blood-vessels of the tunica propria to be further transported to the fat depots.

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SUMMARY

1. The free border of the intestinal absorptive cells of cyprinids is nearly similar to that in tetrapods. This border has been described for four other species of fish and the possible evolution of the border from a ciliated epithelium has been described.

2. The mitochondria of the absorptive cells are arranged in a supranuclear group of mainly rod-like bodies and a subnuclear group of mainly granular ones.

3. The mitochondria are coarser and the Golgi element more readily impregnated with osmium tetroxide in the carnivorous *Gobio* than in the omnivorous *Rutilus* or the herbivorous *Cyprinus*. In all three fish both organoids are associated with fat absorption.

4. The goblet cells secrete both mucus and zymogen. Their stomata are permanently open.

5. Granular cells are described in *Rutilus* and *Cyprinus*, and in seven other species of fish, but they are absent from *Gobio*. Their varying reactions to numerous cytological techniques are described.

6. Alkaline phosphatase is especially concentrated in the free border of the absorptive cells. It occurs along the whole length of the gut. Its possible role in the absorption of fat and glucose is discussed.

7. Lipase is secreted by the absorptive cells. Fat absorption occurs along the whole length of the intestine, but the second limb of the intestine is especially active in this respect.

8. Carbohydrases and lipases are more concentrated towards the anterior end of the gut, whereas proteinases are more abundant caudally.

9. Carbohydrases are richer in *Cyprinus* and *Rutilus* than in *Gobio*.

10. Zymogen and lipase secretions are more concentrated in *Gobio* (carnivorous) than in the other two species.

11. The pH of the intestine of all three fishes is round about neutral point. Free HCl is absent from all three.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. A composite drawing of the intestinal epithelium of *G. gobio* giving a synthetic picture of the cytoarchitecture as revealed by various techniques.

Fig. 2. Vertical section of the intestinal epithelium of *R. rutilus* showing the canal layer of the free border. Ciaccio's technique. $\times 2,100$.

Fig. 3. Tangential section of the free border of *R. rutilus* showing the intercellular bands. Fixative: M.A.S. of Baker; stain: Heidenhain's haematoxylin-cosin. $\times 2,100$.

Fig. 4. *R. rutilus*; two adjacent folds in contact with one another and their free borders greatly expanded. Note canals, intercellular bands and supranuclear group of mitochondria. Champy-Kull method. $\times 1,500$.

Fig. 5. Vertical section of the intestinal epithelium of *R. rutilus* showing intercellular bands. Fixative: M.A.S. of Baker; stain: Masson's trichrome. $\times 1,500$.

Fig. 6. Vertical section of the intestinal epithelium of *Pterois volitans*, showing the free border. Fixative: Zenker-formol; stain: Masson's trichrome. $\times 1,500$.

Fig. 7. Vertical section of the duodenal epithelium of *Scarus sordidus* showing the free border. Fixative: Regaud's; stain: Bensley-Cowdry. $\times 1,500$.

PLATE II

Fig. 8. Vertical section of the intestinal epithelium of *G. gobio* showing the Golgi element (G.E.) and nucleus (N.) of the absorptive cells. Kolatchev's method. $\times 2,100$.

Fig. 9. Vertical section of the intestinal epithelium of *G. gobio* showing a goblet cell (G.C.) and its Golgi element (G.E.) and nucleus (N.). Kolatchev's method. $\times 2,100$.

Fig. 10. *Atherina forskali*, section of submucosa, showing the granular cells. Fixative: Bouin; stain: haematoxylin-eosin. $\times 1,200$.

Fig. 11. *R. rutilus* showing the granular cells. Fixative: Bouin; stain: haematoxylin-eosin. $\times 1,200$.

PLATE III

Fig. 12. *R. rutilus*, section of submucosa, showing granular cells. Fixative: alcohol-formol; stained for glycogen by McManus's method. $\times 1,200$.

Fig. 13. *R. rutilus*, section of submucosa showing granular cells containing alkaline phosphatase. Gomori's method. $\times 850$.

Fig. 14. Transverse section of intestinal wall of *G. gobio* showing location of alkaline phosphatase. Gomori's method. $\times 85$.

Fig. 15. Vertical section of a few absorptive cells of *R. rutilus* showing location of alkaline phosphatase. Gomori's method. $\times 1,200$. Note phosphatase located in Golgi zone, indicated by arrow.

Fig. 16. Transverse section of a part of a fold of the intestine of *G. gobio* showing location of lipase. Gomori's method counterstained lightly with haematoxylin and eosin. $\times 620$.

PLATE IV

Fig. 17. Vertical section of the mucous membrane of the pharynx of *G. gobio* showing location of alkaline phosphatase. Gomori's method. $\times 620$.

Fig. 18. Vertical section of a few absorptive cells from the intestinal mucosa of *R. rutilus* during active digestion. Fixative: alcohol-formol; stain: haematoxylin-mucicarmine. $\times 1,200$. Note the presence of a vacuole (v) in the luminal half of each cell.

Fig. 19. Vertical section of a few absorptive cells from the mucosa of the second limb of the intestine of *R. rutilus*, showing fat globules. Schridde's method. $\times 1,000$ (see Protocol 1, p. 341).

Fig. 20. Transverse section of the apex of an intestinal fold of the intestine of *R. rutilus* showing fat globules. Schridde's method. $\times 1,000$ (see Protocol 2, p. 341).

ABBREVIATIONS USED IN PLATES

C.L. canal layer.
C.L.Z. clear zone of cytoplasm.
G.C. goblet cell.
G.C.M. goblet cell showing mucus.
G.C.Z. goblet cell showing zymogen.
G.E. Golgi element.
GR.L. granular layer.
I.B. intercellular band.
L.M. lymphocyte.
N. nucleus.

P.C.G. 'pear-shaped' cell showing Golgi element.
P.C.R. 'pear-shaped' cell showing rods.
S.C. stratum compactum.
SB.M. subnuclear group of mitochondria.
SF.L. superficial layer.
SG.L. subgranular layer.
SP.M. supranuclear group of mitochondria.
V. vacuole.

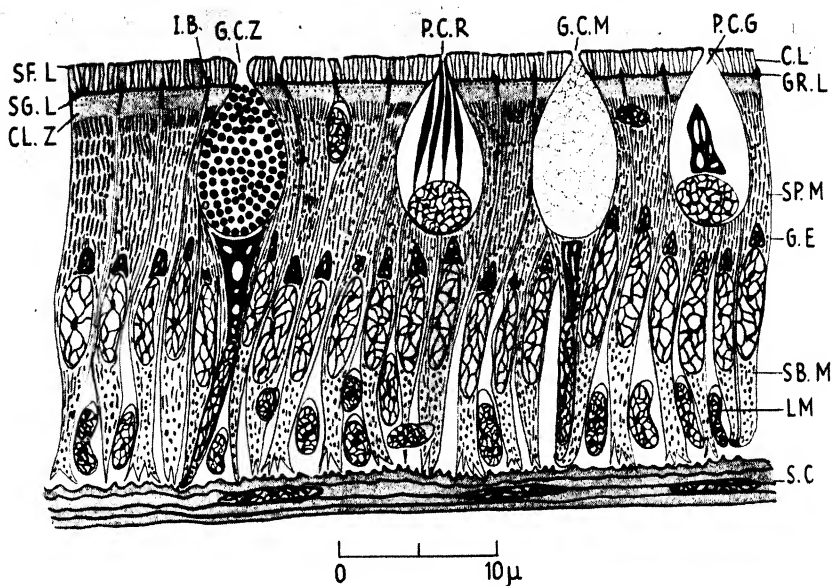
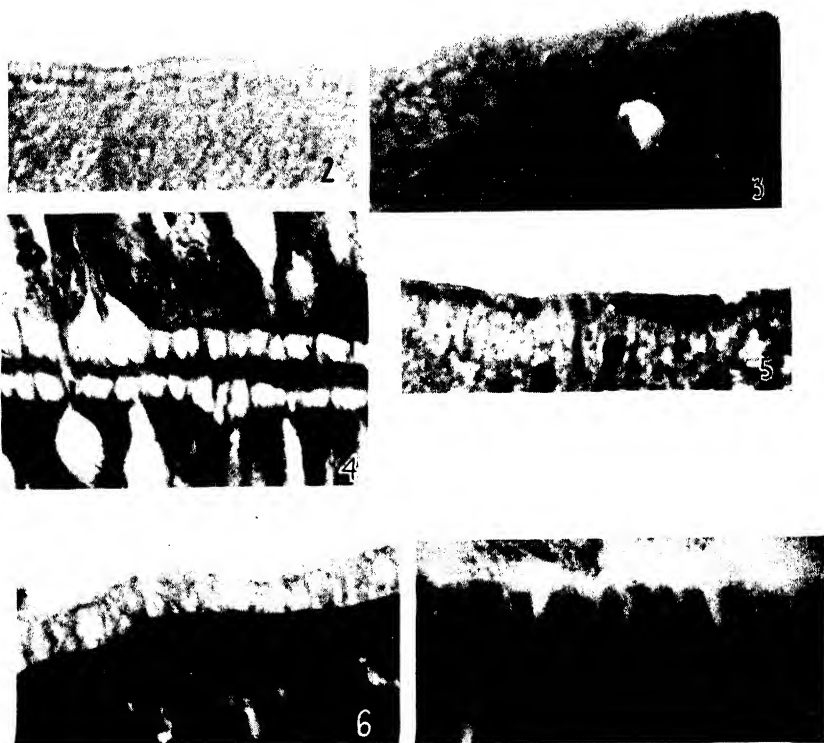
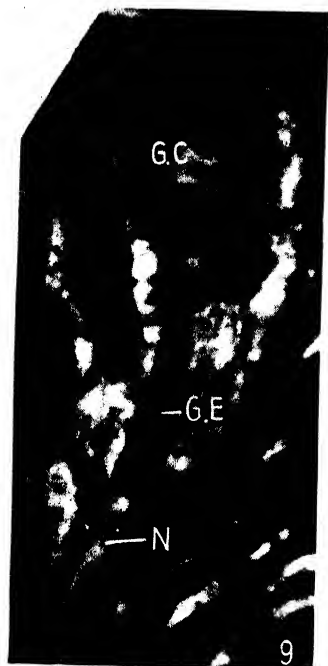
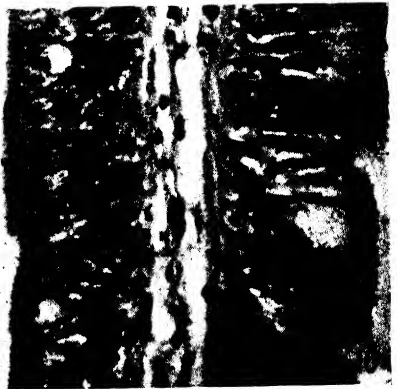
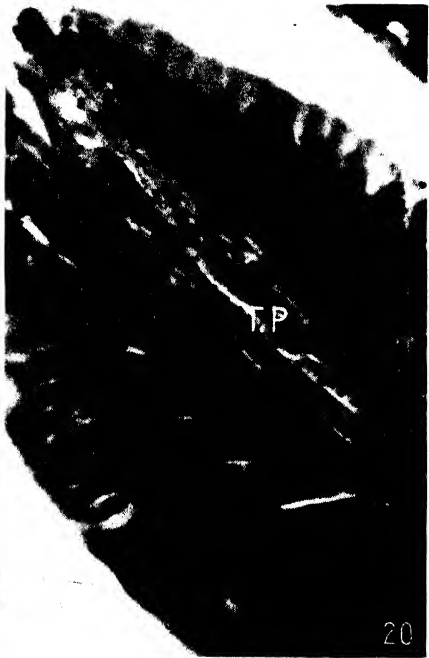


FIG. 1









Mitosis in the Mouse: A Study of Living and Fixed Cells in Tissue Cultures

BY

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With eight Plates

INTRODUCTION

BY means of phase contrast ciné-photomicrography applied to tissue cultures it has become possible to study the division of living vertebrate cells in much greater detail than hitherto. Observations have already been made by these methods on mitosis in chick cells (Hughes and Swann, 1948; Hughes and Fell, 1949) and in amphibian tissue (Hughes and Preston, 1949). The present authors have extended these investigations to mammalian cells, using cultures of new-born mouse tissues, mainly of the spleen and kidney. In this material prophase can be identified in the living cultures at a very early stage and mitosis followed to its completion. Mouse cultures are characterized by a wide variation in cell size; investigations showed that the larger cells are polyploid. The first part of this paper describes the course of normal mitosis and is based on the study of both living and fixed preparations; the second part records the evidence for the polyploid nature of the large nuclei.

Normal mitotic cell division in tissue cultures has been investigated by several authors. One of the earliest accounts is that of Strangeways (1924) who made warm-stage observations on mitosis in chick fibroblasts. The earlier work on mitosis in tissue culture has been fully reviewed by Levi (1934). Some more recent studies by W. H. Lewis (1940) are discussed in the text.

The literature on the chromosome cytology of the mouse has been cited by Grüneberg (1943); the most recent publication on this subject is that of Matthey (1936). In *Mus musculus* the diploid number of chromosomes is 40 and the attachment to the spindle (centromere) is subterminal.

In their research H. B. F. was responsible for the tissue culture and the preparation and study of the fixed material. The photography was by A. F. H. and the analysis of the film records was the joint work of both authors.

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MATERIAL AND METHODS

Tissue Culture

Tissue fragments from the spleen, kidney, and heart of mice killed 3 to 21 days after birth were explanted in large hanging drop cultures on No. 2, 1¼-in. square coverslips which were sealed with molten paraffin-wax to 3×1½-in. hollow-ground slides. The culture medium consisted of one drop of fowl plasma mixed with one drop of 10 to 12-day chick embryo extract; in the later experiments the plasma was diluted with an equal volume of Tyrode before use, so as to provide a softer clot and thus facilitate the emigration of the cells. The cultures were transferred to fresh medium at 3- to 4-day intervals.

After several subcultures the tissue was transplanted to a No. 1 coverslip and embedded in a plasma embryo extract clot in the usual way. A square hole was then cut in the clot, one side of the square coinciding with the edge of the tissue; fluid from the clot immediately exuded into the space. The coverslip was mounted on the special metal chamber described by Hughes and Swann (1948) and the preparation was placed in the incubator. When the tissue began to grow, the outgrowth extended into the clot on three sides, but on the fourth side it advanced over the glass in a thin, expanded sheet of cells covered by the fluid medium in the square hole. Mitosis was active in this thin layer of cells which were excellent for optical study. For phase-contrast photography this simple device was a great improvement both on cultivation in a complete clot in which the emigrating cells are in many different planes and where there is optical interference from the fibrin network, and on cultivation in an entirely fluid medium which makes the preparation awkward to handle and in which the least disturbance or change in temperature may cause a complete or partial retraction of the outgrowth.

Fixation and Staining

The cultures were fixed for a few minutes either in Maximow's fluid¹ or in 2 per cent. osmium tetroxide, usually after being rinsed in warm Tyrode; it is important carefully to drain off the surplus saline in order to ensure instantaneous coagulation of the cells. Tissue cultures are ideal material for cytological purposes, because their extreme thinness allows of almost perfect fixation (Pl. I, fig. 2). The cultures were washed for some hours in several changes of distilled water and were stored for not more than a few days in 80 per cent. alcohol.

Nearly all the cultures were stained by Feulgen's method, after which the central explant was removed leaving only the zone of outgrowth; this was done to reduce the total thickness of the final preparation and so ensure critical illumination during microscopical study. The cultures were counterstained in a mixture of 0.1 per cent. light green+0.1 per cent. naphthol green in 90 per cent. alcohol, and after being dehydrated and cleared in xylene, were mounted whole in Canada balsam or Gurr's DePeX mountant on very thin

¹ 10 parts Zenker's solution; 1 part formal; 1 part 2 per cent. osmium tetroxide.

slides. A few preparations were made in which the cultures were hydrolysed as for Feulgen's method and then stained in well-ripened Ehrlich's haematoxylin (Hughes and Fell, 1949).

Photography

The photographic records of the cultures were made at two magnifications. Fields of up to 100 cells were photographed at a total magnification of $\times 50$. These low-power pictures were taken on 35-mm. film with apparatus designed and used by the late Dr. R. G. Canti and loaned by the British Empire Cancer Campaign. The Canti machine is now installed at the Strangeways Laboratory and has been modified for phase-contrast photography. The records were made on Kodak 'Microfile' film with a $\times 10$ phase-contrast objective; the source of light was a ribbon filament lamp, with a green filter. The interval between exposures was either 1 or $2\frac{1}{2}$ minutes; photography was continued for 2 days.

Single cells were photographed at high magnification on 16-mm. film by a technique which has already been described in detail (Hughes, 1949). Usually a cell in early prophase was chosen and was followed through mitosis for 1-4 hours. The interval between photographs varied from 1 to 6 seconds.

All the objectives and condensers used were manufactured by Messrs. Cooke, Troughton & Simms, Ltd.

Analysis of Film Records

The analysis of the film records is one of the most important and difficult parts of the cinematographic technique. Usually projection at 16 frames per second is too rapid for the detailed study of particular features in cellular processes. On the other hand, the observation of individual frames one at a time is inadequate, as projection in motion is necessary to appreciate the interrelation of adjacent structures.

Recently a 'Craig' Projecto-Editor, generously presented to the Laboratory by the Rockefeller Foundation, has proved of the utmost value in film analysis. With this instrument motion projection at any speed in either direction can be combined with the viewing of single frames. In our opinion this is a necessary piece of apparatus to obtain the best results from film records.

Comparison of the Living with the Fixed Nucleus

In some cultures the same cell was photographed in three different ways: (1) by phase contrast in life, (2) by phase contrast after fixation in 2 per cent. osmium tetroxide, and (3) by ordinary illumination after being stained by either Feulgen's method or Ehrlich's haematoxylin. The procedure was repeated with cells at different stages of mitosis. Two sets of such photographs are shown in Pl. I.

Fixation with osmium produces an almost negligible amount of distortion of the nucleus as is shown by the photographs of an interphase nucleus on Pl. I, fig. 2. The chromocentres and chromonemata (see p. 359 below) are in

focus in both fig. 2a and fig. 2b, Pl. I, and occupy identical positions in both photographs. After fixation the Feulgen-positive chromocentres are no longer visible by phase contrast, but of course reappear when the cell is stained. The same behaviour is shown by the chromosomes during mitosis. In Pl. I, fig. 1a, a spleen cell was photographed at the first moment of anaphase; by the time it had been fixed the daughter groups of chromosomes had separated to the extent shown in Pl. I, fig. 1c. In Pl. I, fig. 1b, taken by phase contrast after fixation, the chromosomes are invisible and the whole spindle area is lighter than the surrounding cytoplasm, densely packed with mitochondria. The radial arrangement of the latter at the poles of the spindle is shown in both fig. 1a and fig. 1b, Pl. I.

INTERPHASE

General Appearance of the Cells

Most of the observations were made on the reticular cells of spleen cultures. When fragments of the spleen are first explanted large numbers of lymphocytes and monocytes emerge; these are followed more slowly by the reticular cells which predominate over other cell types after several passages. They are very suitable for phase-contrast photography as they spread out thinly on the glass and divide actively. The fibroblasts in heart and kidney cultures were also studied but less extensively.

Spleen reticular cells and kidney fibroblasts are of very similar appearance in cultures; the cells of both types vary considerably in size. When the cells are growing in a fluid medium on the surface of the glass (see p. 356), the membranous border of the cytoplasm is often actively motile when seen in a speeded-up film, and may show pinocytosis (Lewis, 1931), i.e. the ingestion of globules of the external fluid which pass into the cell as a stream of clear vacuoles.

The marginal cytoplasm is often quite clear except for the filamentous mitochondria which are exquisitely seen under the phase microscope. Nearer the nucleus fatty globules and, especially in the spleen cells, particles of ingested matter are aggregated round the cytocentre. If too abundant these granular inclusions may obscure the details of mitosis, particularly in the large polyploid and binucleate cells (see below), as they cause a glare in the phase microscope due to phase reversal.

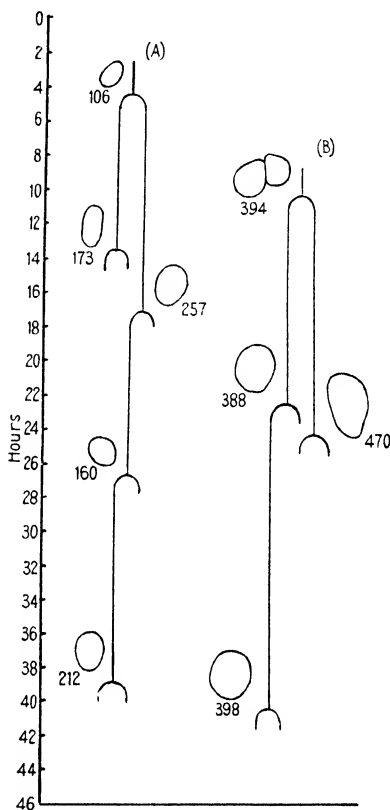
The cytocentre is sometimes hidden by the large granules, but it is often visible in life as a grey, finely granular area to one side of the nucleus. In some cells it has a clearly defined, rounded outline as in a binucleate cell figured by Hughes in a previous paper (Hughes, 1949; Pl. II, fig. 3).

Nuclear Cytology

In both living and fixed cultures, the nuclear structure of spleen reticular cells or kidney fibroblasts is beautifully clear in those cells which are expanded flat on the coverglass. The interphase nucleus, which is usually oval or

rounded, contains from 3 to 7 nucleoli varying greatly in size and shape. In life the nucleoli appear dark grey by phase contrast and do not change their form and relative positions; sometimes they contain vacuoles similar to those described by Lewis (1943).

Feulgen-positive heterochromatic granules, the chromocentres, which look almost black in the living cell, are stuck to the nucleoli and to the inner surface of the nuclear membrane; a similar appearance has been described by Ludford, Smiles, and Welch (1948) in mouse tumour cells. These chromocentres range in number from about 10 to 72; when only a few are present they are much larger than when there are many, which suggests that the large bodies are formed by the fusion of several small heterochromatic granules. In Table I are listed the number of chromocentres in 21 interphase nuclei. Five nuclei (marked) were much bigger than the rest; one of them had only 25 chromocentres which, however, were very large, but the remaining 4 contained many (36–72). In Feulgen preparations of well-fixed cells the ground substance of the nucleus, which stains pale pink, seems almost homogeneous at first sight. Close inspection with a high-power apochromat, however, shows it to consist of a mass of faintly granular filaments, the chromonemata, which are particularly clear in cultures fixed with 2 per cent. osmium tetroxide (Pl. II, fig. 4). The chromonemata are attached to the chromocentres and are always more distinct near the point of attachment than elsewhere; parts of the chromonemata are visible in life in a large, well-flattened cell (cf. Ludford *et al.*, 1948), especially near the union of the threads with the chromocentres. There appears to be considerable variation in the visibility of chromocentres and chromonemata in interphase nuclei. When searching in living cultures for cells in early prophase we have often found nuclei containing obvious filaments and granules and have followed them for



TEXT-FIG. 1. Diagram of cell lineages from a spleen culture, photographed at low power. Each A represents a mitosis, and the vertical lines intermitotic periods. For each parent nucleus in early prophase the outline is given, together with the nuclear area in square microns. (a) Lineage of a cell with a medium-sized nucleus. (b) Lineage of a binucleate cell, the descendants of which had large nuclei.

than elsewhere; parts of the chromonemata are visible in life in a large, well-flattened cell (cf. Ludford *et al.*, 1948), especially near the union of the threads with the chromocentres. There appears to be considerable variation in the visibility of chromocentres and chromonemata in interphase nuclei. When searching in living cultures for cells in early prophase we have often found nuclei containing obvious filaments and granules and have followed them for

as long as 1 hour, during which time they remained unchanged. Such nuclei may possibly be approaching mitosis.

TABLE 1. *Table showing the Variation in the Number of Chromocentres in Different Nuclei*

K = kidney culture. Those unmarked are spleen cultures.
L.M. = large mononucleate cell.

<i>Interphase</i>	<i>Prophase</i>	<i>Daughter nucleus</i>
10	30	20
14	32	22
16	38	22
16	39	22
K 17	40	K 23
18	40	24
K 19	K 44	27
21	44	31
23	48	35 L.M.
K 25	54	K 54 L.M.
25 L.M.
26
27
28
28
29
34
K 36 L.M.
K 42 L.M.
62 L.M.
72 L.M.

The Intermitotic Period

From 6 low-power records of the growth of mouse spleen cultures one film which covered a period of 50 hours was selected for intensive study. When photography began there were about 20 cells in the field, and when the record ended there were about 70. Fifty-one mitoses were counted which were remarkably evenly spaced throughout the 50 hours.

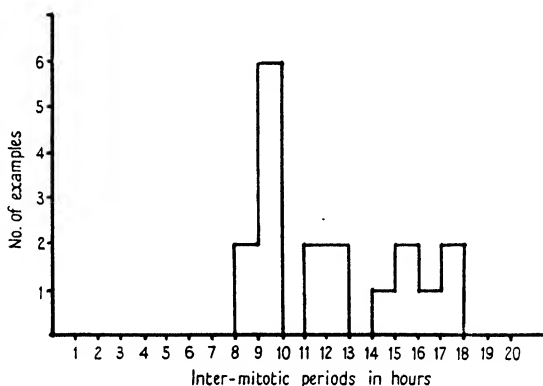
Whenever possible the daughter cells produced by each division were traced forward through the film until they entered their next division or could no longer be identified. Eighteen intermitotic periods were thus recorded, and one cell was followed through four generations (Text-fig. 1). The intermitotic intervals ranged from 6 to 18 hours with a mode of 9 hours (Text-fig. 2). There was no correlation between the size of a nucleus at prophase and the time which elapsed before its daughter cells divided.

MITOSIS

I. Diploid Mitosis

The onset of *prophase* is characterized by an increase in the number and a diminution in the size of the heterochromatic granules (Pl. II, figs. 5 and 6;

Table 1). The chromonemata seen in the interphase nucleus become much more distinct and are now clearly visible in the living cell, though at this stage they are less sharply defined with phase contrast than are the mitochondria (Pl. V, fig. 31*a*). As the nucleic acid charge on the chromosomes increases the heterochromatic granules can no longer be counted accurately, as they cannot always be distinguished with certainty from bends and kinks in the long threads. The bends and kinks diminish, however, as the threads contract and the picture becomes much less confused.



TEXT-FIG. 2. Histogram showing distribution of intermitotic periods. Data from the same spleen culture as in Text-fig. 1.

Some of the chromosomes radiate from the nucleoli (cf. Lewis, 1940), but many are applied to the inner surface of the nuclear membrane where their structural details can be studied. One end of each thread is occupied by one of the heterochromatic granules which stains more intensely with Feulgen's method than the rest of the chromosome (Pl. II, fig. 7) and behind which the thread is often seen to be split; in a living chromosome the terminal heterochromatic granule is well shown. The chromosomes often appear finely banded both in the living nucleus and in Feulgen preparations (Pl. II, fig. 8); possibly the bands represent gyres in the contracting thread.

Whether all the chromosomes terminate in a heterochromatic granule is uncertain, as it is only occasionally possible to find a free end unobscured by neighbouring threads. It can be stated, however, that whenever a heterochromatic region can be clearly observed it is found to occupy the end of a chromosome, and that when both ends of the same chromosome can be distinguished only one has a heterochromatic granule. It is also uncertain whether the heterochromatic region is near the centromere, which is sub-terminal in the mouse, or whether it forms the distal end of the chromosome.

A direct morphological continuity between the mitotic chromosomes and the chromocentres of the interphase nucleus was observed in living cells in

tissue culture by W. H. Lewis (1940). This author, however, does not distinguish between the Feulgen-positive chromocentres and the Feulgen-negative nucleoli and applies the latter term to all intranuclear granules. It seems clear, however, that the 'small nucleoli' of Lewis which were seen to be in structural continuity with certain chromosomes correspond to the chromocentres in our material, and that the chromosomes which seemed to develop from the large nucleoli in his cells were, in fact, associated with the chromocentres sticking to the surface of the nucleoli.

Our films show that in early prophase the nucleus often revolves. The rotation usually occurs in greatly expanded cells and seems to be associated with the spindle, since the asters often appear shortly after it has taken place. It is possible that the nucleus is rotated by the spindle material forcing its way between the nuclear and cell membranes, which in very flattened cells are separated by a mere film of cytoplasm. As the chromosomes acquire their nucleic acid charge the heterochromatic region ceases to be distinguishable, and the longitudinal split dividing each chromosome into two chromatids is now obvious in stained cultures (Pl. II, fig. 8). In the living cell the split in the chromosomes is usually first seen nearer metaphase (Pl. V, fig. 31c).

In favourable cells the formation of the asters can be followed in some detail (Pl. V, fig. 31b); the process is particularly well seen in polyploid cells (Pl. VI, fig. 32c). They first appear at the poles of the early prophase nucleus as rounded areas of dark granular cytoplasm similar to that of the cytocentre of interphase. In these areas the mitochondria and other cell inclusions soon acquire a radial orientation. The spindle is formed from the asters and sometimes each half of the developing spindle produces a deep indentation of the nuclear membrane (Pl. VI, fig. 32c). A few seconds later the nuclear membrane suddenly collapses and disappears, the nucleoli finally vanish and the cell enters *metaphase* (Pl. V, fig. 31c; Pl. II, figs. 10 and 11). At the time when the nuclear membrane vanishes the whole cell contracts. The extended processes are withdrawn, and the surface area is reduced. In the living cell the spindle is seen as a clear area surrounded by dark, rather granular cytoplasm (Pl. V, fig. 31d). The chromatids of each pair are now clearly separated except at the centromere (Pl. II, fig. 10), and for several minutes they move at random up and down the spindle (cf. Lewis, 1939; Hughes and Swann, 1948). In mouse cells the radial stage, characteristic of early metaphase in chick fibroblast cultures (Hughes and Fell, 1949), is only occasionally seen (Pl. II, fig. 9). In the chick cells the spindle probably forms at right angles to the plane of the cover-slip and then rotates through 90° until it is parallel with the cover-slip, whereas in the mouse cells the spindle usually develops in the plane of the cover-glass from the beginning and there is no subsequent rotation. When a radial arrangement of the chromosomes is seen in exceptional mouse cells the asters are not visible, being above and below the metaphase plate.

The chromosomes soon become orientated near the equator, and after an interval of 6 to 20 minutes *anaphase* begins (Pl. V, fig. 31f; Pl. II, fig. 12); the

chromatids separate and pass to opposite poles of the spindle (Pl. V, fig. 31g, h). A few minutes later the cytoplasm begins to constrict at the equator (Pl. V, fig. 31h, i) and the cell enters *telophase*. Both anaphase and telophase are accompanied by the usual cytoplasmic 'bubbling'.

Close attention was paid to the process of *nuclear reconstruction* as it is shown in Feulgen preparations, in the films, and by direct observation of the living cells. The development of a living daughter nucleus from telophase until the end of the first few hours of interphase is illustrated in Pl. VII, fig. 34; the area of this nucleus (see p. 367) is plotted against time in Text-fig. 3.

As soon as the daughter chromosomes reach the poles they shorten and become closely packed together (Pl. V, fig. 31h, and Pl. II, fig. 13). Suddenly this chromosomal mass begins to swell; at the same time it unravels into a web of fine threads radiating in all directions from the oblong areas of refractile, chromatinic material which represent the original chromosome bodies (Pl. VII, fig. 34c) and which in fixed preparations are still strongly Feulgen-positive (Pl. II, figs. 14, 15).

In the film records it is sometimes possible to follow for a short time the transformation of one or two individual daughter chromosomes in the compact group. The chromosome becomes very short and plump, then vacuoles appear in its substance and it rapidly dilates (cf. Lewis, 1940); at the same time a delicate lattice-work of threads, closely associated with that of neighbouring chromosomes, is drawn out of the shrinking chromatinic material. At the interface between the chromosome and the cytoplasm, a sector of the nuclear membrane develops.

In Feulgen preparations the vacuoles which form in the interior of the chromosomes make them appear double (Pl. II, fig. 15), but we are doubtful whether this apparent doubleness does, in fact, represent a split in preparation for the next mitosis. The problem is being investigated further.

At first the young nucleus is a narrow body with an irregular outline, but as it continues to swell it assumes a smooth, oval contour (Pl. VII, fig. 34c). The chromatinic areas become less refractile in the living cell and in fixed preparations are seen to have lost much of their Feulgen positive charge, except in the terminal heterochromatic segment (Pl. IV, fig. 25). They are rapidly replaced, however, by a more diffuse system of irregular lumps and strands which occupy the greater part of the nucleus and which are much more clearly seen in the living nucleus (Pl. VII, fig. 34e, f) by phase contrast than in Feulgen preparations. The subsequent history of this system shows that it represents the nucleolar material.

When the film records are traced backwards, the nucleolar material seems to form at the sites of the original chromosomes, but the close study of an unusually favourable record indicated that the chromatic material disappears before the nucleolar substance becomes visible. The surface of the developing nucleoli is studded with dark heterochromatic granules from each of which fine filaments emerge, so that the nucleolar components are knit together by an intricate mesh of threads (Pl. VII, fig. 34d, g). Other heterochromatic

granules adhere to the nuclear membrane. It is noteworthy that throughout the nucleus most of the threads appear taut, as if under tension. The heterochromatic granules are intensely Feulgen-positive in fixed preparations; although most of the chromosomes have now shed nearly all their nucleic acid charge, a few of those attached to the nucleoli lost it much more slowly than the others.

The larger nucleolar masses form where the greatest number of chromosomes are aggregated. As time goes on, the nucleoli become even more refractile and less diffuse (Pl. VII, fig. 34g, *h*).

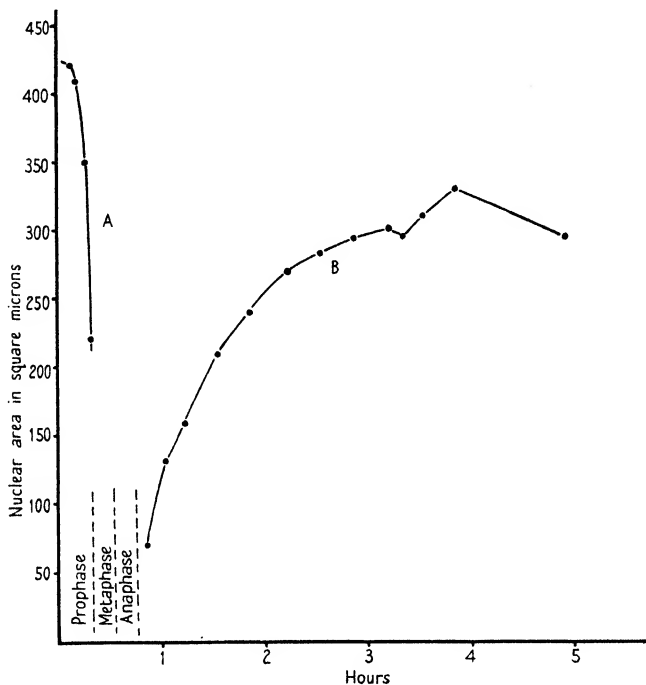
Although the nucleus continues to swell, during the later stages of reconstruction there is little, if any, increase in size of the nucleoli, which therefore occupy a diminishing proportion of the nuclear volume. As a result, there is an extensive rearrangement of the nuclear contents (Pl. VII, fig. 34i-k) which is interesting to watch in the film records. Sometimes two nucleolar masses which were originally close together move apart and the chromosome threads connecting them are then drawn out to several times their previous length; elsewhere the nucleolar substance may retract from the margin of the nucleus, leaving behind a chromocentre stuck to the nuclear membrane. Meanwhile the nucleoli change their appearance; as they become more refractile, the diffuse, straggling nucleolar system of the young daughter nucleus becomes transformed into the compact, rather rounded masses characteristic of interphase (Pl. VII, fig. 34k). At the same time the chromosome threads become less conspicuous and the chromocentres to which they are attached more distinct both in life and in Feulgen preparations (Pl. IV, fig. 27). Finally the nucleus ceases to enlarge and the mature interphase condition has been reached (Text-fig. 3).

We tentatively suggest the following interpretation of some of the appearances seen in nuclear reconstruction. In early telophase the compactly arranged chromosomes swell, presumably by imbibition of water, and are thus brought into even more intimate contact than before. The nuclear membrane forms at the interface between the chromosomes and the cytoplasm; the nucleus then rapidly dilates and the chromosomes are forced apart by the expansion. At the same time the chromosomes begin to lose their nucleic acid charge and their structure becomes looser, as indicated by the small vacuoles which appear within them (cf. Wilson, 1925, pp. 134-8). The chromosomes seem to adhere to each other, to the nuclear membrane, and to the developing nucleoli, so that when they are thrust apart by the dilation of the nucleus, it is possible that their complex adhesions may cause loops of their chromonemata to be dragged out in all directions by the intranuclear tensions and thus form the delicate web of threads so beautifully seen in the living as well as in the fixed cell (Pl. IV, fig. 25; Pl. VII, fig. 34d); as mentioned above, the general straightness of the threads suggests that they are being held taut by mechanical forces. It must be emphasized that this explanation of the arrangement of the fine filaments is mere hypothesis as the fibres are too near the limits of resolution for their detailed structural relations to be determined by ordinary microscopical observation.

II. Heteroploid Mitosis

The spleen reticular cells and kidney fibroblasts in the zone of outgrowth may be classified in three groups:

1. Mononucleate cells of medium size which are probably diploid and which form the greater part of the cell population (Pl. II, figs. 3 and 4).



TEXT-FIG. 3. Nuclear area of a cell during mitosis, (a) that of the parent cell in prophase, (b) that of one daughter cell in telophase and early interphase (this daughter cell is shown in Pl. VII).

2. Large mononucleate cells which appear to have more than the normal number of chromosomes, i.e. to be heteroploid (Pl. III, figs. 16, 18, and 19).
3. Binucleate cells with either medium or large nuclei (Pl. III, fig. 17).

The observations we are about to describe suggest that these groups are not distinct cell types but are produced by variations in the mitotic process.

1. *The formation of a binucleate cell.* Of 51 mitoses recorded in a spleen culture photographed at low magnification for 50 hours, one produced a binucleate cell (Pl. IV, fig. 30, compare Pl. IV, figs. 24 and 29). Division was apparently normal, but cytoplasmic cleavage was not completed and the daughter cells reunited (cf. Strangeways, 1924; Fell and Andrews, 1927).

If the incidence of mitosis without effective cleavage is of the order of 1 in 50, as this record would suggest, the rate of formation would not account for all the binucleate cells seen in the outgrowth during a single 3- to 4-day passage. It is probable that many binucleate cells survived from previous passages and some may have emigrated from the explant.

In normal mitosis the daughter cells often remain connected by the interzonal strand for some time (Pl. V, fig. 31j) and while such continuity persists, reunion is always possible. Binucleate cells can be artificially produced by exposing a dividing cell to strong illumination which often suppresses cleavage completely.

2. *Mitosis in binucleate cells.* Six records of binucleate cells in prophase have been obtained, in all of which one spindle was formed and both sets of chromosomes joined a single metaphase plate (Pl. VIII, figs. 36 and 37). One of the cells was fixed at this stage. In two records anaphase was followed by cleavage and two abnormally large mononucleate daughter cells resulted (Pl. VIII, fig. 36); one of the films was made under low magnification and the daughter cells were followed through two subsequent generations of mononucleate cells (see p. 360 and Text-fig. 1).

In three of the records, mitosis did not end in cleavage and a binucleate cell was again formed. The chromosomes were very distinct in one of these cells which contained few cytoplasmic granules (Pl. VIII, fig. 37). They became arranged on a metaphase plate where they remained for 35 minutes; an imperfect anaphase ensued in which the chromatids moved an abnormally short distance apart and in which one half of the spindle seemed to be defective, since both groups shifted in the same direction across the field. During the next half-hour daughter nuclei were reconstructed from the tetraploid groups of chromosomes. In this way a cell with two diploid nuclei was converted into one with two tetraploid nuclei.

In a fixed preparation a cell was encountered containing two separate spindles each with a metaphase plate, but unfortunately we have no ciné records of mitosis of this type. There may be other unrecorded variants of mitosis in binucleate cells.

We have no evidence of the fusion of nuclei in binucleate cells during interphase. Very large nuclei containing many nucleoli have occasionally been seen in which the nuclear membrane showed an equatorial infolding which might have represented a stage in the fusion of two nuclei; although one such nucleus was watched for several hours it gave no indication that fusion was in progress.

3. *Mitosis in large mononucleate cells.* Several records were made of the division of large mononucleate cells similar to those which may result from the fusion of a binucleate element. The appearance of the nuclei at prophase suggests that they contain more than the diploid number of chromosomes (Pl. VI, fig. 32a and b).

In living cells, several details at this stage were more clearly seen in heteroploid than in diploid elements. Thanks to the larger size of the former, it was

easily possible to focus separately on the interior of the nucleus and at the level of the nuclear membrane (Pl. VI, figs. 33*b* and *c*). The position of chromonemata and chromocentres on the inner surface of the nuclear membrane was thus quite clear. The asters appeared either in polar indentations of the nuclear membrane as described above (Pl. VI, fig. 32*c*), or asymmetrically to one side of the nucleus (Pl. VIII, fig. 35*c*).

Between prophase and metaphase the movements of the chromosomes in relation to the asters and spindle were clearly seen in these large nuclei (Pl. VI, fig. 32 *c-f*). Before the nuclear membrane had vanished the chromosomes crowded towards the two poles, giving a spurious effect of anaphase (Pl. VIII, fig. 35*b*; Pl. III, fig. 21). When the membrane had gone and the spindle had formed, the numerous and rather widely scattered chromosomes moved towards the spindle (Pl. VI, figs. 32*c-d*). These facts suggest that the chromosomes are drawn in some way towards the asters and spindle. The spindle was of normal size, and during metaphase seemed over-crowded with chromosomes. Anaphase, telophase, and cleavage proceeded normally in such cells, and two large daughter cells were produced in the example shown in Pl. VIII, fig. 35.

Attempts were made to count the chromosomes in fixed and stained preparations of these large cells. Tissue cultures are very unfavourable for chromosome counts, however, because as already stated the metaphase plate usually lies at right angles to the plane of the coverslip. All our counts were almost certainly too low, but they showed that many more than the diploid number of chromosomes were present.

4. *Observations on nuclear size.* The results described above implied that in cells of the same histological type, the size of the nucleus, at the same point in the mitotic cycle, is related to the number of its chromosomes (compare Pl. II, fig. 10, and Pl. IV, fig. 22; also in Pl. IV, fig. 25 with fig. 26, and fig. 27 with fig. 28).

The areas of a number of living nuclei in early prophase, including some of the largest, were measured in the following way. From each of twenty-two records of mitotic cells made at high magnification, a frame in prophase was selected before the nuclear membrane had begun to contract. This was projected at a total magnification of $\times 2,000$, and the area bounded by the nuclear membrane was measured with a planimeter. The nuclear volume could not be estimated as it is impracticable to measure the depth of so thin a structure as the nucleus of a flattened tissue culture cell. It is reasonable to assume, however, that a relationship exists between the volume and the area of the projected nuclear image, which for convenience is termed 'the nuclear area'. The measurements were restricted to well-flattened spleen reticular cells.

The results are listed in Table 2. It will be seen that the nuclear areas range from 198 to 625 sq. μ . The difference in volume between the large and medium-sized nuclei is greater than the projected areas suggest, since the large prophase nuclei are thicker than the smaller ones, as shown by the fact

that in the former it is possible to focus separately on the nuclear membrane and internally at the level of the nucleoli.

TABLE 2. *Data from 19 Records of Mitosis in Cells of Mouse Spleen*

The area of the nucleus in prophase at the beginning of the record and the duration of each phase of division is given.

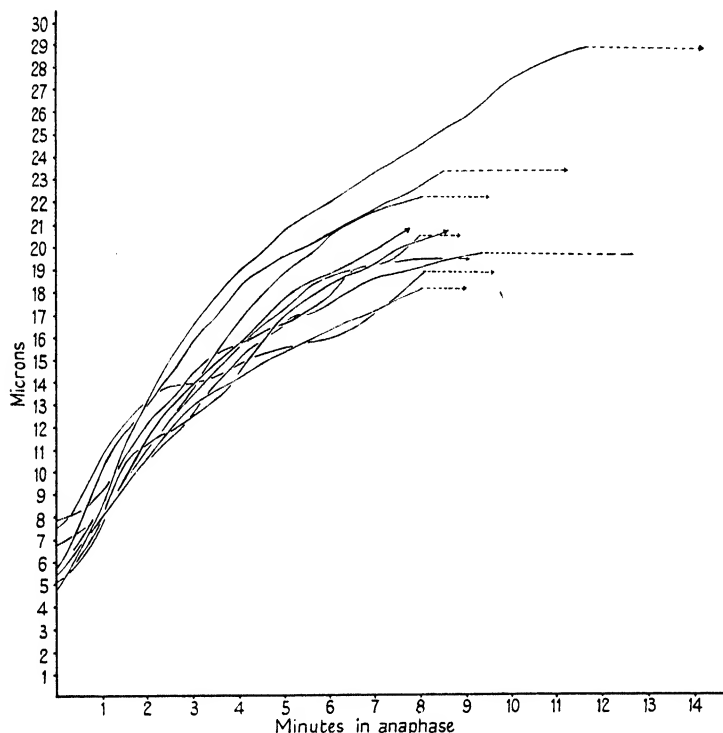
Record no.	Nuclear area in prophase in square microns	Time of phases in minutes							Type of cell
		Sequence begins in prophase	Nucleoli and membrane go	Metaphase begins	Anaphase begins	Cleavage furrow appears	Cleavage ends	Daughter nucleoli evident	
1	198	13.2	2.1	15.3	5.0	3.8	9.3		Medium mono-nucleate
2	215	5.9	5.8	11.6	4.4	4.1	9.7		
3	229	4.0	4.1	19.8	3.9	5.6	13.8		
4	249	12.8	5.8	20.0	3.5	5.5	24.4		
5	255	5.9	5.8	11.6	4.4	4.1	9.7		
6	270	30.0	5.5	18.0	4.8	4.8	20.4		
7	272	19.0	10.0	7.4	6.6	← 12.0 →			
8	296	19.8	13.3	5.7	5.3	2.4	15.6		
9	315	21.4	6.6	12.0	5.0	4.0	20.3		
10	333	18.8	4.4	9.4	5.4	8.7	26.3		
11	420	5.1	4.4	18.6	4.9	5.4	40.0		Large mono-nucleate
12	451	2.8	6.5	15.5	5.7	6.5	13.0		
13	465	21.0	11.1	14.0	7.7	6.8	4.2		
14	465	..	8.2	3.9	6.4	7.3	11.2		
15	575	8.0	4.3	> 14.8		
16	625	37.8		
17	330 (both)	10.6	9.3	17.5	6.1	9.2	24.2		Binucleate with cleavage
18	384 (both)	20.0	← 103.0 →						No cleavage
19	443 (both)	3.0	6.0	35.0	← 39.0 →				

The following observations show that a difference in nuclear size between two cells of the same culture may persist in their descendants through several generations. As already stated, one of the low-power ciné records was analysed exhaustively and a few cell families were traced through several mitoses. The nuclear areas at prophase were measured throughout two of these lineages; the parent of one family was a medium-sized mononucleate reticular cell, and that of the other a binucleate cell of the same type, to which reference has already been made (p. 366), and in which the two interphase nuclei fused in late prophase.

Selected frames of the film were projected at a total magnification of $\times 600$, and the outlines of the nuclei were traced on squared paper. The data thus

obtained are given in Text-fig. 1, and show clearly that the descendants of the mononucleate cell all have smaller nuclei than those of the binucleate element.

The results obtained from the measurements of living nuclei further support the view that the medium-sized nuclei are approximately diploid and that the large ones are polyploid. Probably there is considerable variation in chromosome number between the precisely diploid and the polyploid states.



TEXT-FIG. 4. Anaphase curves of a group of ten cells with medium-sized nuclei. The dotted extension of the curve is continued to the point of cleavage of the cell and indicates that the chromosome groups became indistinct in early reconstruction before this point.

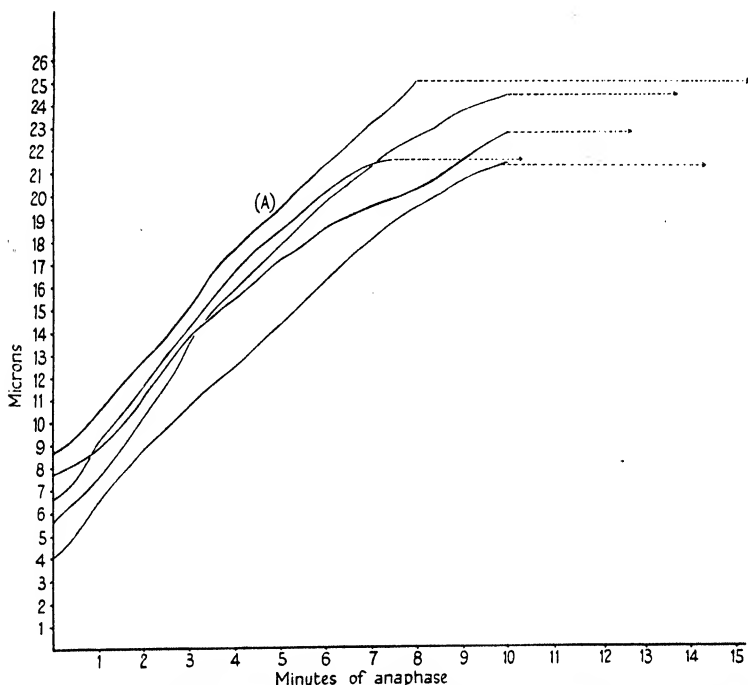
The combined areas of the nuclei of binucleate cells in prophase in Table 2 is intermediate between the areas of the nuclei in the two groups of mononucleate cells. It is possible that the nuclei of binucleate cells are generally aneuploid.

III. Anaphase Movement

The rate of movement of the chromosomes during anaphase has been measured from the film records, and the results are plotted in Text-figs. 4 and 5. The distance between the daughter chromosomes is measured from

the axial centromeres, i.e. from the surfaces of the chromosome groups which face the spindle poles. The duration of the cytoplasmic cleavage is indicated in Text-figs. 4 and 5; after this point nuclear reconstruction had begun, so that the distance measured no longer represents that between the centromeres.

Text-fig. 4 refers to anaphase movement in a group of ten cells with prophase nuclei of medium size. Text-fig. 5 shows the anaphase curves of four



TEXT-FIG. 5. Anaphase curves of a binucleate cell (A), and of a group of four large mononucleate cells. The dotted lines have the same significance as in Text-fig. 4.

cells with large, presumably polyploid nuclei and of one binucleate cell. A comparison of the two figures indicates that the rate of movement in the normal and in the large cells falls within the same range; this implies that the spindle is the same size in both groups, a conclusion that is supported by direct measurements of the distance between the spindle poles. Cleavage of the cytoplasm proceeds more slowly in polyploid and binucleate cells than in diploid elements, probably owing to the larger size of the former.

The anaphase curves in the mouse-tissue cultures are similar in shape to those of amphibian cells *in vitro* (Hughes and Preston, 1949). In both there is first a sigmoid inflexion, and the maximum velocity is reached 2-3 minutes from the beginning of anaphase, after which it steadily declines. From the

curves of diploid anaphase (Text-fig. 4), an average curve was calculated giving a maximum chromosome velocity of 1.8μ per minute; in *Rana* and *Xenopus* the maximum velocity is about 2.0μ per minute. The final distance between the daughter chromosome groups is similar in *Mus*, *Rana*, and *Xenopus*. The period from the beginning of anaphase to the end of cleavage in *Mus* is intermediate between that in *Rana* and *Xenopus*.

In the chick (Hughes and Swann, 1948) the initial sigmoid inflexion is very small and the daughter chromosomes attain their maximum velocity of 4μ per minute almost as soon as their movement can be accurately measured. Two possible reasons may be suggested for these differences between anaphase movement in the chick and in other vertebrates. The tearing apart of the long chromosome arms is amphibian and mouse mitosis may exert a drag on the centromeres at early anaphase and thus retard their motion; alternatively the spindle may be a better orientated structure in chick cells than in those of other animals. It is unlikely that the maximum rate of anaphase movement is governed by the size of the chromosomes, because in Amphibia, chromosomes of widely different lengths move together with their centromeres ranged on a regular arc.

IV. Duration of Mitotic Phases

The duration of each phase of division was calculated from the ciné records and compared with measurements made from direct observation of the living cells during photography. The results are expressed in Table 2.

In Table 2 the mitotic process is subdivided into six stages which, however, cannot be defined with equal precision. As the cell was already in prophase when photography began, the record of this phase of division is incomplete and the longest period recorded is therefore the nearest approach to the true value. By the disappearance of the nucleoli is meant that stage in prophase when the chromosomes attached to the nucleoli become more conspicuous than the nucleolar material itself; these chromosomes remain associated for some minutes longer, however, and it is probable that the nucleoli do not finally disappear until the chromosomes become free. The beginning of anaphase can be determined to within one or two frames of the film. The end of cleavage is usually sharply marked at the time when equatorial constriction ceases, although an isthmus of variable size may connect the daughter cells for some time. The most difficult point to define is that when the nucleoli and chromocentres first become discrete structures; as described above, the nucleolar material is first distinguishable early in telophase before the chromosomes have completely shed their nucleic acid charge.

In Table 2 the total time occupied by division minus the duration of prophase which has to be estimated (see above) is recorded for nineteen cells which are arranged in three groups according to the type of mitosis which they undergo (see p. 365). In group I, the smaller mononucleate cells, mitosis proceeds faster than in the other groups. There is a larger scatter in the duration of each period in the large mononucleate cells of group II than in the

smaller cells. The cycle of chromosome changes is slowest in the non-dividing binucleate cells of group III.

DISCUSSION

Different methods of observation have led to divergent views on the structure of the nucleus during interphase. The doctrine of chromosome continuity between successive divisions, although never doubted, was not securely based on direct observation. Although the earlier cytologists described the structure of the interphase nucleus as they saw it in fixed and stained preparations, in terms of chromosome continuity, their views on nuclear structure were not accepted by students of the living cell. Thus Lewis and Lewis (1924) state that in the nuclei of living cells in tissue culture 'no linin network or chromatin granules are to be seen. These are fixation, coagulation, or precipitation products and do not represent living structures.'

This opinion, which was shared by most of those who worked with living tissue cultures, was based on the fact that when living cells in cultures are examined by ordinary direct illumination or by the dark-field method, only the nucleoli are visible and the ground substance appears optically homogeneous. Moreover, M. H. Lewis showed that living cells could be gelated by treatment with dilute acid which produced first a granulation and then a coarse reticulum in the nucleoplasm; under appropriate conditions this gelation could be reversed, when the granules and reticulum disappeared. These interesting experiments naturally led to the conclusion that similar appearances in fixed preparations were artifacts and should be disregarded.

Use of the phase-contrast method suggests that the structures described by earlier cytologists in fixed cells were only partly artificial. The statement that the ground substance of the living nucleus is optically homogeneous is due to the limitations of the microscopical methods previously available. It is well known that unstained structures are visible under the microscope by virtue of differences in refractive index between them and their surroundings. Both the ordinary bright-field and the dark-field microscopes are insensitive to small gradations of refraction. The dark-field microscope brilliantly reveals sharp discontinuities of structure of which the nuclear membrane is an example; as a result the nuclear membrane scatters the converging rays of the dark-field condenser so much that the fine detail in the nucleus does not deflect enough light to be recognized.

These optical limitations of the bright and dark fields are largely overcome by using the phase microscope. As described above, in the living interphase nucleus studied by this means, three types of structure are visible: (1) the nucleoli, (2) small dark granules, some adherent to the nucleoli and others to the inner surface of the nuclear membrane, and (3) fine threads attached to the small granules; the threads, which are the least conspicuous objects in the living nucleus, are seen best in a young cell which has recently divided. When the same nucleus which has been observed and photographed in life is fixed with osmium tetroxide and stained by Feulgen's method, very little distortion

is produced; the small dark granules in the living nucleus are found to correspond with the Feulgen-positive chromocentres, and the delicate threads emerging from them are seen to form part of an intricate mass of chromonemata which in the Feulgen-stained nucleus are seen completely to fill the space between the nucleoli and the nuclear membrane.

In the well-fixed cells composing the thin zone of outgrowth, the filamentous structure in the nuclear ground substance is only revealed by careful scrutiny under the most critical optical conditions. On the other hand, many of the interphase nuclei left sticking to the glass after removal of the explant are less perfectly fixed, owing to the slower penetration of the reagent in this part of the culture, and these often show a fine network resembling the linin reticulum described by earlier workers. In our view, this appearance is caused by the defective fixation of the chromonemata which have become irregularly clotted together instead of being evenly distributed in the nucleus in the normal way. Thus the linin network of the earlier observers may be regarded as a real cytological entity artificially distorted by fixation. Bensley (1933) showed that nuclei fixed by the freeze-drying technique were similar in appearance to those treated with chemical fixatives, and concluded that the structures seen in stained preparations of the nuclei were not mere fixative artifacts.

The structure and cytochemistry of the resting nucleus has recently been investigated by Serra (1947) who concluded that 'mitotic chromosomes usually arise from resting nuclei of the thread type, that is from nuclei in which threads which take up chromatin dyes and give strong Feulgen and arginine reactions are conspicuous'.

The state of the chromosomes in the interphase nucleus has recently been investigated by Ris and Mirsky (1949) who, like the earlier workers on tissue cultures, state that 'In the living interphase nucleus no chromosomal structures are visible' (p. 500). As described above, in the living interphase nuclei of mouse cells grown *in vitro*, phase contrast (not used by Ris and Mirsky) reveals fine threads which we regard as chromonemata; the threads are most distinct in young daughter nuclei (Pl. VII, fig. 34*g* and *k*). In the mature nucleus they are usually best seen near their union with the chromocentres; precisely the same type of structure is visible in the nuclei after fixation with osmium or Maximow's fluid (Pl. II, figs. 3 and 4); sometimes the same chromonema can be identified in a nucleus before and after fixation. In the fixed nucleus, however, the complete system of chromonemata is distinguishable. The threads are faintly Feulgen-positive, and a small amount of desoxyribonucleic acid may have been deposited on them during fixation, as described by Ris and Mirsky for fixatives other than osmium. As recorded above, in the mouse cells during telophase each chromosome rapidly swells and thus enters the 'extended state' of Ris and Mirsky, but in our material the chromonemata became clearly visible during the swelling process (see p. 363).

In certain plants the number of Feulgen-positive granules or chromocentres in the nucleus is approximately the same as that of the chromosomes (Wilson, 1925). Overton (1909) named them 'prochromosomes' and suggested

that they were aggregates of residual chromatin around which the chromosomes were formed early in prophase. Heitz (1928) showed that the pro-chromosomes were segments of the mitotic chromosomes, which retained the full charge of nucleic acid in interphase. These segments were called 'heterochromatic'. Recent literature on the nature and function of 'heterochromatin' has been reviewed by Schultz (1947). The chromocentres of human tumour cells have been studied by Koller (1947) and those of mouse carcinomata by Biesele (1944*b*).

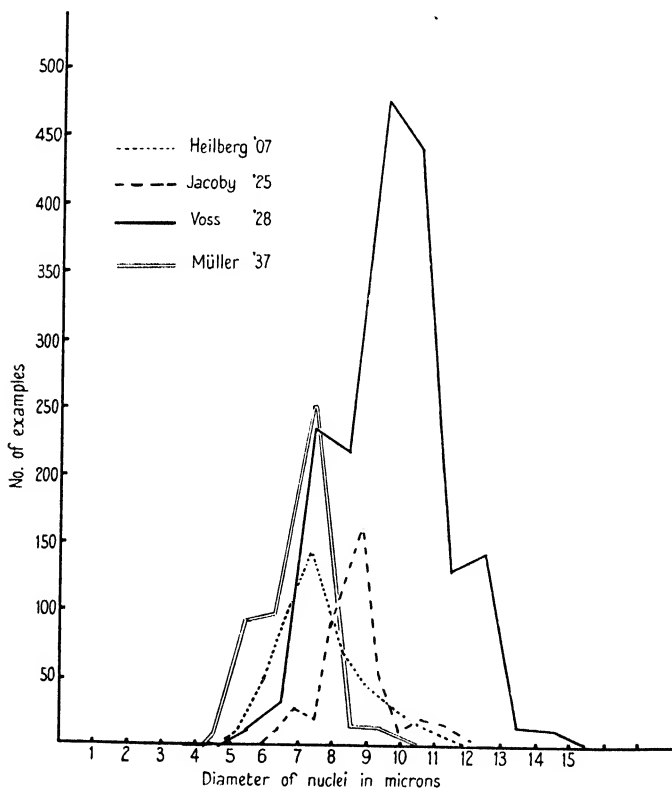
A striking feature of the chromocentres is their apparent adhesiveness; they are always attached to the surface of either the nucleoli or the nuclear membrane. This characteristic, which has also been noted by Vanderlyn (1946), is very conspicuous in the living mouse nuclei viewed with the phase microscope. There is almost no depth of focus with the phase microscope, so that it is possible to focus separately on a number of planes even in so thin a structure as the nucleus of a tissue-culture cell. On the surface of the nuclear membrane the mitochondria are seen, and a very slight movement of the fine adjustment reveals the chromonemata and chromocentres adherent to the inner surface of the membrane. In a large polyploid nucleus in early prophase, the focus can then be shifted to the level of the nucleoli, to which other chromocentres are attached (Pl. VI, fig. 33*b* and *c*). This distribution of the chromocentres is less obvious in fixed and stained preparations, as fixation and dehydration shrink the nucleus in a direction vertical to the coverslip. The nucleolar chromocentres remain stuck to the dwindling nucleoli late in prophase. The chromonemata become well charged with desoxyribonucleic acid before the nucleoli disappear, so that towards the end of prophase chromosomes radiate from the nucleoli in the manner described by Lewis (1940).

If the heterochromatic segment of the chromosomes is adjacent to the centromere, the attachment of the chromocentres to the nuclear membrane or nucleolus might be regarded as equivalent to the attachment of the chromosomes to the spindle. Unfortunately, however, we have been unable to find conclusive evidence as to which end of the chromosome is occupied by the heterochromatic segment.

As described above, the so-called euchromatic region of the chromosome, which is adjacent to the heterochromatic segment, is reduced in interphase to the residual chromonema by the loss of most of the nucleic acid charge. It is usually believed that this process is accompanied by an uncoiling of the chromonema: 'the change from metaphase to resting nucleus consists spatially in an uncoiling and repacking of the chromosome thread' (Darlington, 1937, p. 34). Conversely, in prophase the chromonema is thought to become coiled as it acquires its charge of nucleic acid. In our material the chromosomes are too small for any coiling or uncoiling to be clearly distinguishable, but, as already stated, the mouse chromosomes in prophase often have a finely banded appearance which may represent the gyres of a fine coil.

A relationship between nuclear size and chromosome content was first noted by Boveri (1905) in haploid, diploid, and tetraploid sea-urchin embryos.

His observations were confirmed by Balzer (1910) and Herbst (1912), and similar conclusions were reached by Artom (1928) who studied diploid and tetraploid races of *Artemia salina*. In Amphibia, heteroploidy has been investigated in great detail and the literature has recently been reviewed by Fankhauser (1945). Heteroploid division has also been described in normal



TEXT-FIG. 6. Histograms of the nuclear diameter of cells of the mouse liver, extracted from the data given by the authors indicated.

mammalian cells. It has been seen in human spermatogonia and spermatocytes (Painter, 1923), in the rat liver (Biesele, 1944a) and in the embryonic tissue of the mouse (Howard, personal communication).

The interphase nucleus of normal mammalian cells vary considerably in size and several authors, notably Jacoby (1925, 1935), have attempted to show that in distribution curves of nuclear sizes, there are modes which correspond to simple multiples of nuclear volume (*ibid.*). Nuclear size has been studied in the liver of the mouse by Heilberg (1907), Jacoby (1925), Voss (1928), and Müller (1937). The distribution curves of nuclear diameters derived from the data given by these authors are plotted in Text-fig. 6; it is

hardly possible to identify the modes of these curves with degrees of heteroploidy. In the rat liver also, nuclear sizes have been investigated by Jacoby (1935) and by Beams and King (1942), but the curves obtained in these two studies also fail to agree.

Bieseke (1944a) made chromosome counts in rat liver and found a slight preponderance of diploid over tetraploid nuclei in both the normal and regenerating tissue; the proportion of octoploid nuclei was much smaller. He also observed irregularities in chromosome number which were not exact multiples of the haploid number and which are termed aneuploid. If aneuploid cells are fairly numerous, their presence must contribute to the difficulty of interpreting data on nuclear size. Our own observations suggest that aneuploid nuclei are fairly common in mouse tissue cultures.

Distribution curves of nuclear size can only indicate that a cell population is not uniform and more direct evidence is needed before heterogeneity in volume can be attributed to polyploidy. Such evidence was obtained by Beams and King (1942) in the regenerating liver of the rat. These authors concluded that tetraploid nuclei were formed by the mitosis of binucleate cells. Their description of this process, based on the study of stained preparations, is exactly confirmed by our own observations on living mouse cells *in vitro*. They also describe the formation of a cell with two tetraploid nuclei from one with two diploid nuclei, by mitosis without cleavage; this, too, we have observed in life (see p. 366). Formerly, nuclei of double size in the liver were thought to arise by an amitotic process (Jacoby, 1925, 1935; Müller, 1937), a view which was discarded by Beams and King; we ourselves have never seen amitotic division in vertebrate tissue cultures.

Our own observations on the formation of polyploid cells in tissue cultures were made before we had read the paper by Beams and King. That similar results should have been obtained *in vivo* shows that the variation in the mitotic process whereby polyploid cells are produced is not an abnormality peculiar to tissue cultures. It would be interesting to know whether the origin of polyploid from binucleate cells is a general phenomenon in mammalian tissues. Wherever binucleate and large mononucleate cells occur together, it may well be suspected that the latter originate from the former.

In the Insecta polyploidy is achieved by a quite different process, viz. 'endomitosis'. Thus, in the nurse cells of the ovary of *Drosophila*, the chromosomes split and double within the nuclear membrane during prophase (Painter and Reindorp, 1939). Bieseke (1944b) suggested that in the nuclei of tumour cells chromosomes may double their size by an endomitotic process.

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SUMMARY

1. Mitosis has been investigated in cultures of infant mouse tissues, in both living and fixed material.
2. Phase microscopy combined with serial photography was used to study the living cell.
3. The formation of chromosomes has been traced in the living cell from the intermitotic chromonemata and heterochromatic chromocentres.
4. Dividing cells can be recognized early in prophase and the duration of each phase of mitosis has been measured.
5. Curves of anaphase movement have been plotted; they are sigmoid as in amphibian cells.
6. The reconstruction of the daughter nuclei has been studied in detail in both living and fixed cultures. The chromosomes swell, then begin to lose their nucleic acid charge and to unravel into a web of fine threads. The nucleolar material can be identified in the living cell early in telophase; it appears as a diffuse system of lumps and strands occupying most of the nucleus and its direct transformation into the interphase nucleoli was followed in the film records.
7. The origin of polyploid nuclei by the mitosis of binucleate cells has been demonstrated.
8. Analysis of the film records showed that the average length of the intermitotic period is 12 hours.

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DESCRIPTION OF PLATES

PLATE I

All $\times 2,800$

Fig. 1. Cell in early anaphase (spleen culture).

(a) By phase contrast, living: note the pattern of mitochondria around spindle.

(b) By phase contrast, fixed in OsO_4 : the chromosomes are invisible, but the outline of the spindle is seen.

(c) Stained with the Feulgen reagent.

Fig. 2. Cell in interphase (spleen culture).

(a) By phase contrast, living: within the nucleus the small dark granules are chromocentres and the larger bodies are nucleoli.

(b) By phase contrast, fixed. The chromocentres are almost invisible.

(c) Stained with the Feulgen reagent. Note the very close correspondence of the chromocentres and nucleoli in (a) and (c).

PLATE II

Nuclei in interphase, and in normal diploid mitosis, $\times 2,800$; all except Fig. 3 are Feulgen preparations.

Fig. 3. Intermitotic nucleus (spleen culture). Note the chromonemata in the ground substance. Haematoxylin.

Fig. 4. Intermitotic nucleus (kidney culture), showing nucleoli, chromocentres, and chromonemata.

Fig. 5. Early prophase (spleen culture). The chromonemata are more distinct and the chromocentres smaller and more numerous than at interphase.

Fig. 6. Slightly later prophase (kidney culture). Nucleoli are still present.

Fig. 7. Later prophase (kidney culture). The nucleoli have disappeared. Note the terminal heterochromatic granules (*h*) on the chromosomes.

Fig. 8. End stage of prophase in spleen cell, just before collapse of nuclear membrane. The chromosomes are now obviously double.

Fig. 9. 'Prometaphase' in kidney cell. The nuclear membrane has gone and the chromosomes have contracted. The chromosomes lie in a plane parallel to the coverslip which is rare in mouse-tissue cultures.

Fig. 10. Metaphase in spleen cell. The chromosomes are not yet equatorially arranged.

Fig. 11. Full metaphase in spleen cell.

Fig. 12. Early anaphase in spleen cell.

Fig. 13. Telophase in spleen cell. The chromosomes form a tight mass.

Fig. 14. Daughter nucleus in reconstruction (heart culture).

Fig. 15. Daughter nucleus in slightly later reconstruction (spleen culture). Some of the chromosomes appear double.

PLATE III

Tetraploid and binucleate cells in interphase and mitosis. $\times 2,800$. All Feulgen preparations, except Fig. 21.

- Fig. 16. Tetraploid nucleus in interphase (spleen culture).
 Fig. 17. Binucleate cell in interphase (kidney culture).
 Fig. 18. Tetraploid cell in very early prophase (kidney culture).
 Fig. 19. Tetraploid cell in prophase (kidney culture). The nucleoli have nearly disappeared.
 Fig. 20. Binucleate cell in late prophase (spleen culture).
 Fig. 21. Tetraploid cell at the end of prophase (spleen culture). Haematoxylin. The nuclear membrane has disappeared, and the chromosomes are crowding round the two opposite poles of the spindle (compare Pl. VIII, fig. 37*b*).

PLATE IV

All Feulgen preparations, $\times 2,800$, except Fig. 30.

- Fig. 22. Tetraploid metaphase (spleen culture). Compare with Pl. II, fig. 11.
 Fig. 23. Early anaphase of a tetraploid cell (kidney culture). Compare with Pl. II, fig. 12.
 Fig. 24. Telophase (spleen culture). The daughter nuclei remain within one cell.
 Fig. 25. Reconstruction of a normal diploid daughter nucleus (spleen culture) at a later stage than in Pl. II, fig. 15. Note the fine transverse filaments connecting the chromosomes.
 Fig. 26. Reconstruction of a tetraploid daughter cell at the same stage as in Fig. 25 (kidney culture).
 Fig. 27. Late stage in the reconstruction of a diploid nucleus (kidney culture). The nucleoli and chromocentres are now distinct.
 Fig. 28. Late reconstruction stage of a tetraploid nucleus (kidney culture). Compare with Fig. 27.
 Fig. 29. Late reconstruction stage in a binucleate cell (spleen culture).
 Fig. 30. Stages in the formation of a binucleate cell in a spleen culture, photographed by phase contrast at low power; all enlarged to $\times 220$.
 (a) The upper cell is approaching the end of interphase.
 (b) $2\frac{1}{2}$ hours later. Cell in anaphase.
 (c) 12 min. later than (b). Cleavage apparently complete.
 (d) 28 min. later than (c). Daughter cells flattening, but still connected by a strand.
 (e) 15 min. later than (d). They reunite to form one binucleate cell.

PLATE V

Fig. 31. Mitosis in a diploid cell (spleen culture). Phase contrast enlargements, all $\times 1,500$, from frames of 16 mm. film. Duration of complete record: 74 min.

- (a) Prophase. Nucleoli still present but chromosomes developing.
 (b) 3.7 min. later than (a). The nuclear membrane is shrinking, and is indented by the spindle asters above and below.
 (c) 6.3 min. later than (b). Early metaphase. The spindle is outlined by mitochondrial material.
 (d) 9.7 min. later than (c).
 (e) 3.4 min. later than (d). The split in the metaphase chromosomes is clearly visible.
 (f) 0.9 min. later than (e). The beginning of anaphase.
 (g) 3.1 min. later than (f). Mid-anaphase. The interzonal region of the spindle is being constricted as is shown by the inward movement of mitochondria.
 (h) 4.5 min. later than (g). Orientated mitochondria are seen in the interzonal region. The groups of daughter chromosomes are closely packed.
 (i) 2.5 min. later than (h). Cleavage nearly complete and the groups of daughter chromosomes are swelling.
 (j) 9.8 min. later than (i). The daughter cells are connected by an interzonal strand (s). In the lower daughter the nuclear membrane is visible. The nucleoli are now distinct.
 (k) 13.0 min. later than (j). The young interphase nucleus is increasing in size, and the nucleoli have more contrast.
 (l) 16.7 min. later than (k). Further development of the interphase nucleus.

PLATE VI

Fig. 32. Prophase and metaphase of a polyploid cell (spleen culture). Phase-contrast enlargements, all $\times 1,500$, from frames of 16-mm. film. Duration of record: 25.4 min.

- (a) Prophase. Nucleoli are still present. Compare nuclear size with that in Pl. V, fig. 31a.
- (b) 5.6 min. later than (a). Nucleoli are disappearing.
- (c) 5.9 min. later than (b). The asters have deeply indented the nuclear membrane.
- (d) 1.9 min. later than (c). The nuclear membrane has disappeared; the spindle has been formed and chromosomes are migrating towards it.
- (e) 8 min. later than (d). Many of the chromosomes are not yet on the equator of the spindle.

(f) 4 min. later than (e). The chromosomes are nearer the metaphase arrangement.

Fig. 33. Prophase of a polyploid cell (spleen culture). Phase-contrast enlargements, all $\times 1,500$, from frames of 16-mm. film record. Duration of record: 23 min.

- (a) A polyploid prophase. The plane of focus is within the nucleus, at the level of the nucleoli.
- (b) 14 min. later than (a). Note the change in shape and arrangement of the nucleoli.
- (c) Immediately after (b). The focus has been changed to the level of the nuclear membrane, on the inside surface of which are many chromosome threads.
- (d) 8.8 min. later than (c). The development of the chromosomes is further advanced.

PLATE VII

Fig. 34. Anaphase, telophase, and reconstruction of one daughter nucleus of a polyploid cell in a spleen culture. Phase-contrast enlargements, $\times 2,530$, from frames of 16-mm. film. Duration of complete record: about 5 hours. For description see p. 363. n = nucleolus. ch = chromonema.

- | | |
|---|----------------------------|
| (a) 4 min. after the beginning of anaphase. | (g) 19 min. later than (f) |
| (b) 2.3 min. later than (a). | (h) 60 min. " " (g). |
| (c) 11.1 min. " " (b). | (i) 15 min. " " (h). |
| (d) 6.1 min. " " (c). | (j) 22 min. " " (i). |
| (e) 4.5 min. " " (d). | (k) 24 min. " " (j). |
| (f) 7 min. " " (e). | |

PLATE VIII

Fig. 35. Polyploid nucleus in prophase, to show movement of chromosomes (spleen culture). Phase-contrast enlargements, $\times 1,500$, from frames of 16-mm. film. Duration of record: 17 min.

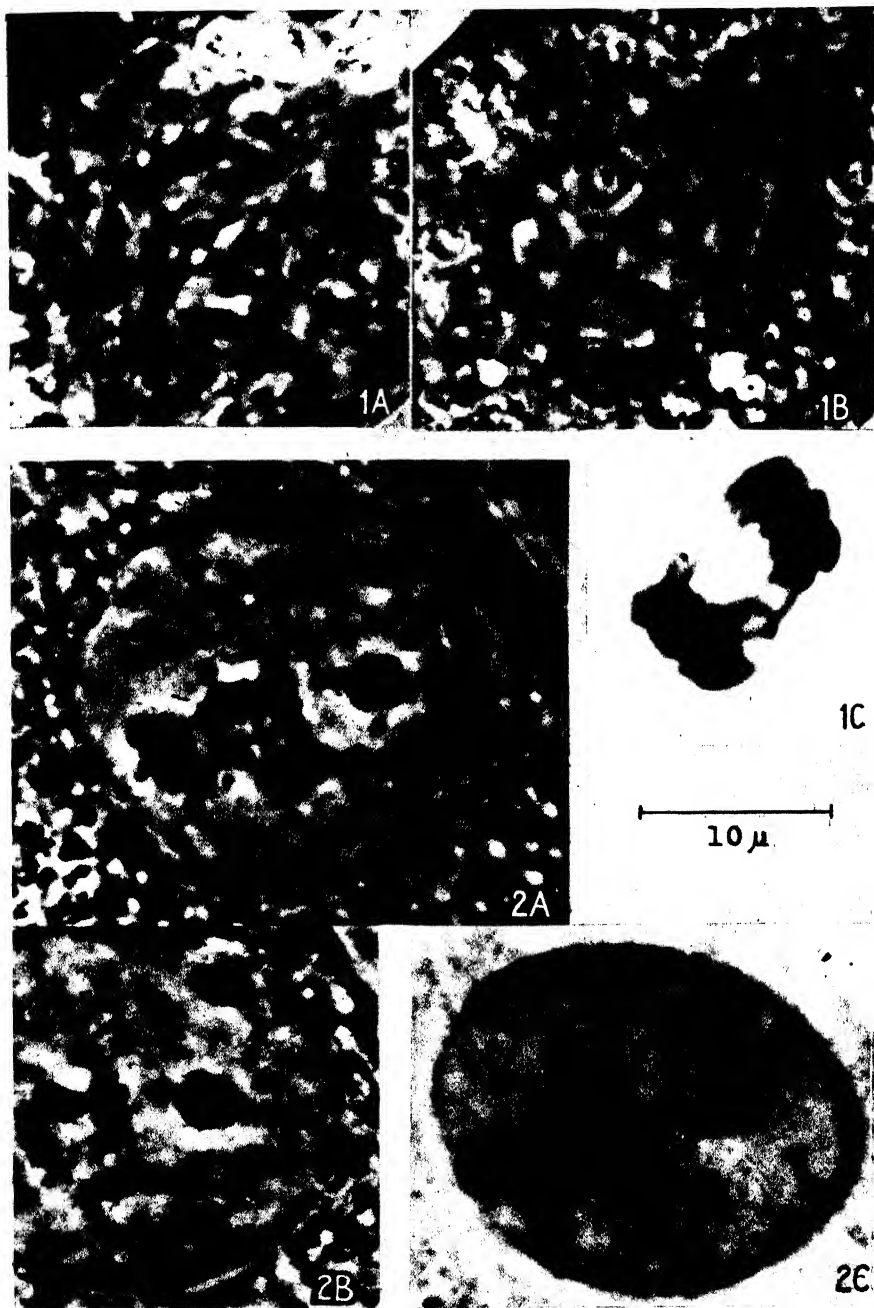
- (a) Late prophase, with nucleoli gone.
- (b) 2 min. later than (a). Nuclear membrane contracting.
- (c) 15 min. later than (b). Most chromosomes have crowded round the two asters, which lie to the left of the nuclear area. A group of chromosomes at the top remain separate, and are not brought on to the metaphase plate until 25 min. later.

Fig. 36. Mitosis of a binucleate cell (spleen culture). Phase-contrast enlargements, $\times 1,500$, from frames of 16-mm. film. Duration of record: 129 min.

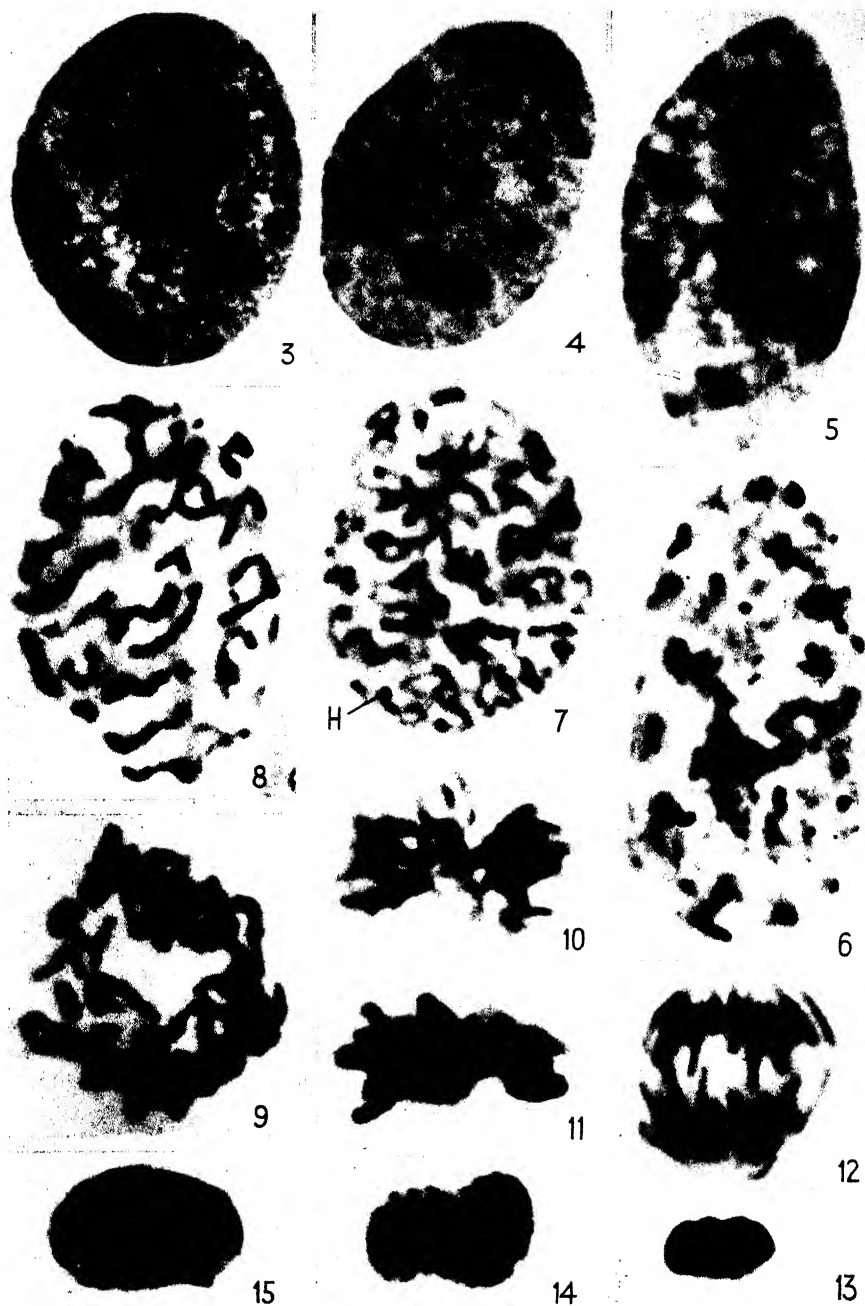
- (a) Late prophase; nucleoli and nuclear membrane still present.
- (b) 9 min. later than (a). Nuclear membranes have gone, and the two diploid sets of chromosomes come together.
- (c) 40 min. later than (b). Anaphase.
- (d) and (e) 80 min. later than (c). The polyploid nucleus of each daughter cell.

Fig. 37. Mitosis of a binucleate cell, without cleavage (spleen culture). Phase-contrast enlargements, $\times 1,500$, from frames of 16-mm. film. Duration of record: 253 min.

- (a) Prophase. Nucleoli and nuclear membranes still present.
- (b) 3 min. later than (a). The nuclear membranes have gone and the diploid chromosome sets have come together.
- (c) 125 min. later than (b). Tetraploid daughter nuclei now nearly as big as the parent diploid nuclei and still in the same cell.
- (d) 125 min. later than (c). Rearrangement of the nucleoli in each nucleus.



H. B. FELL AND A. F. HUGHES—PLATE I





16



19



18



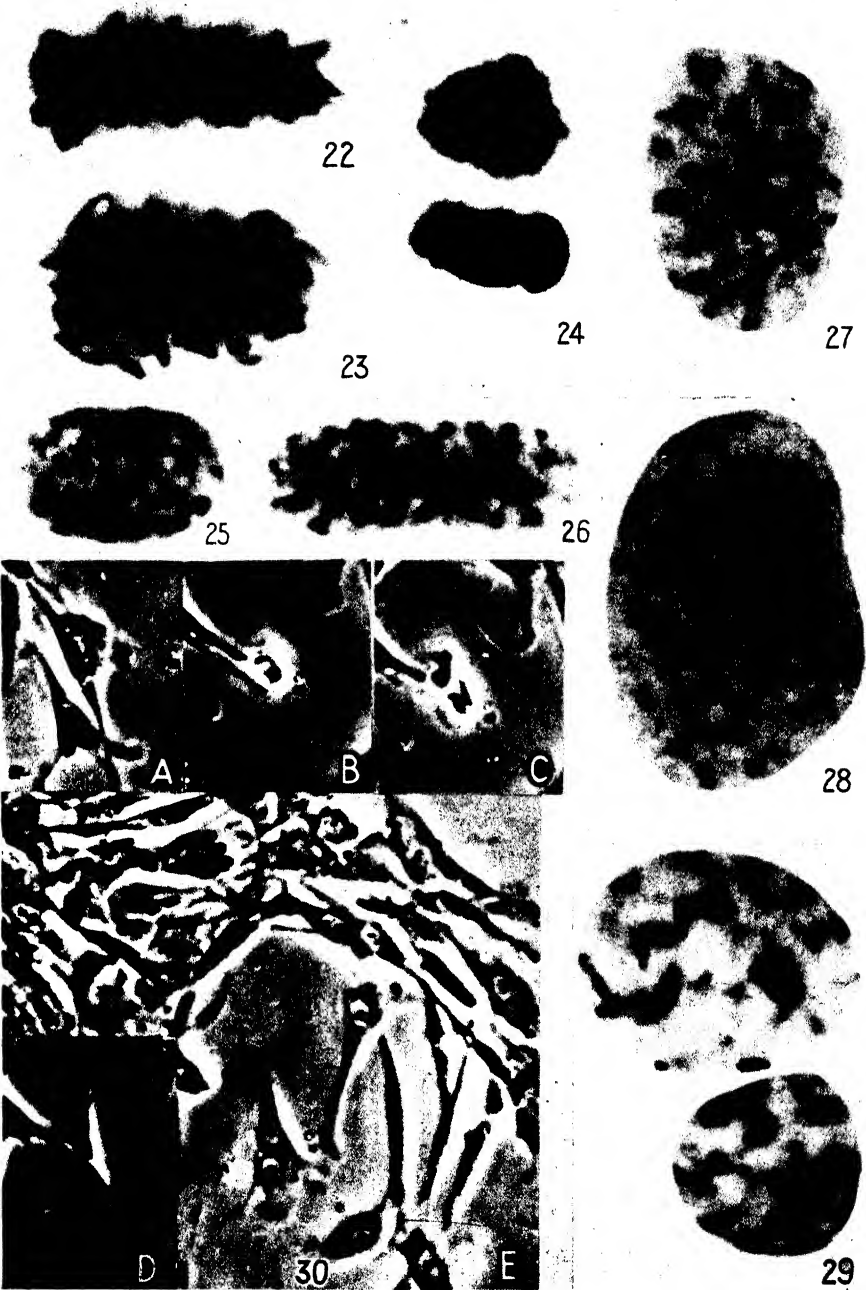
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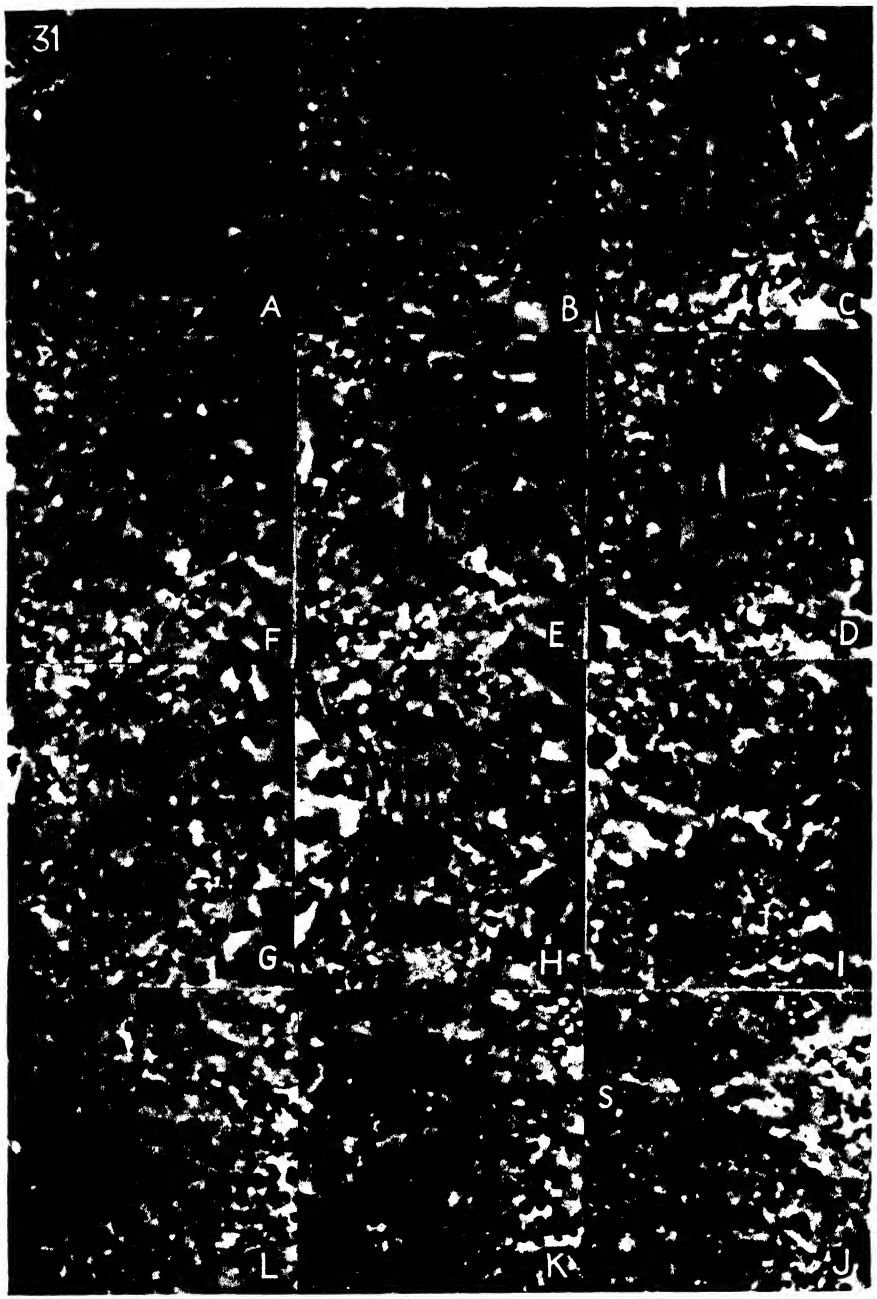
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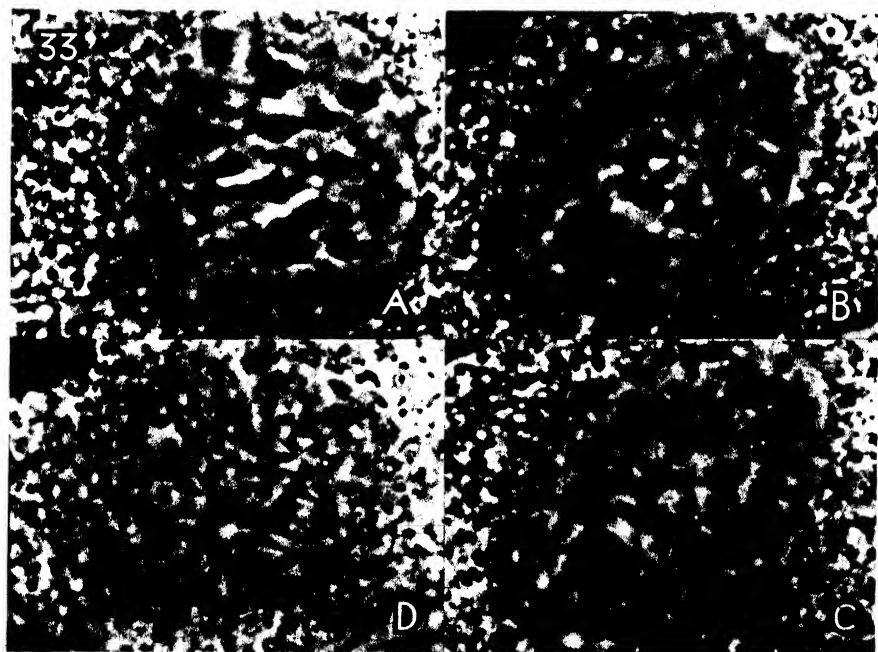
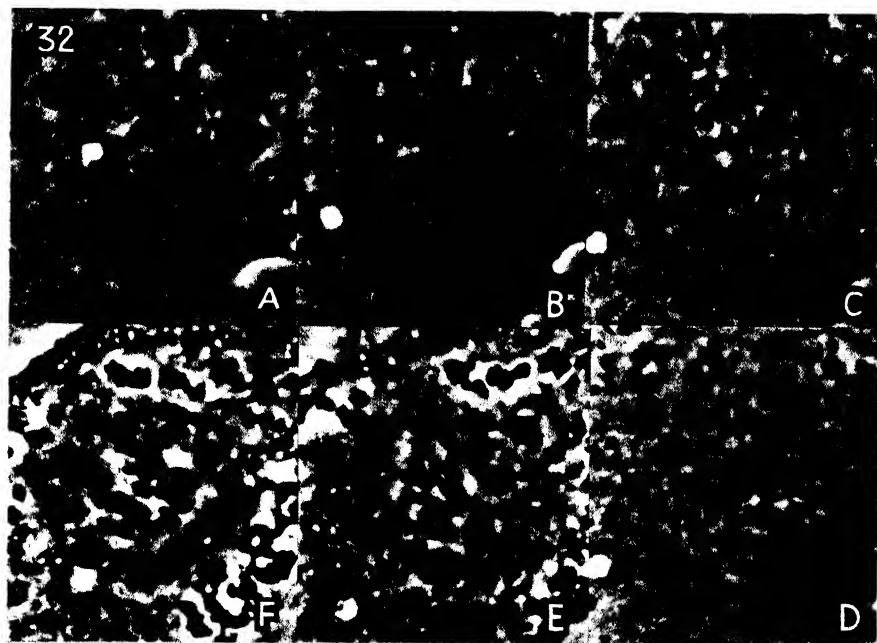
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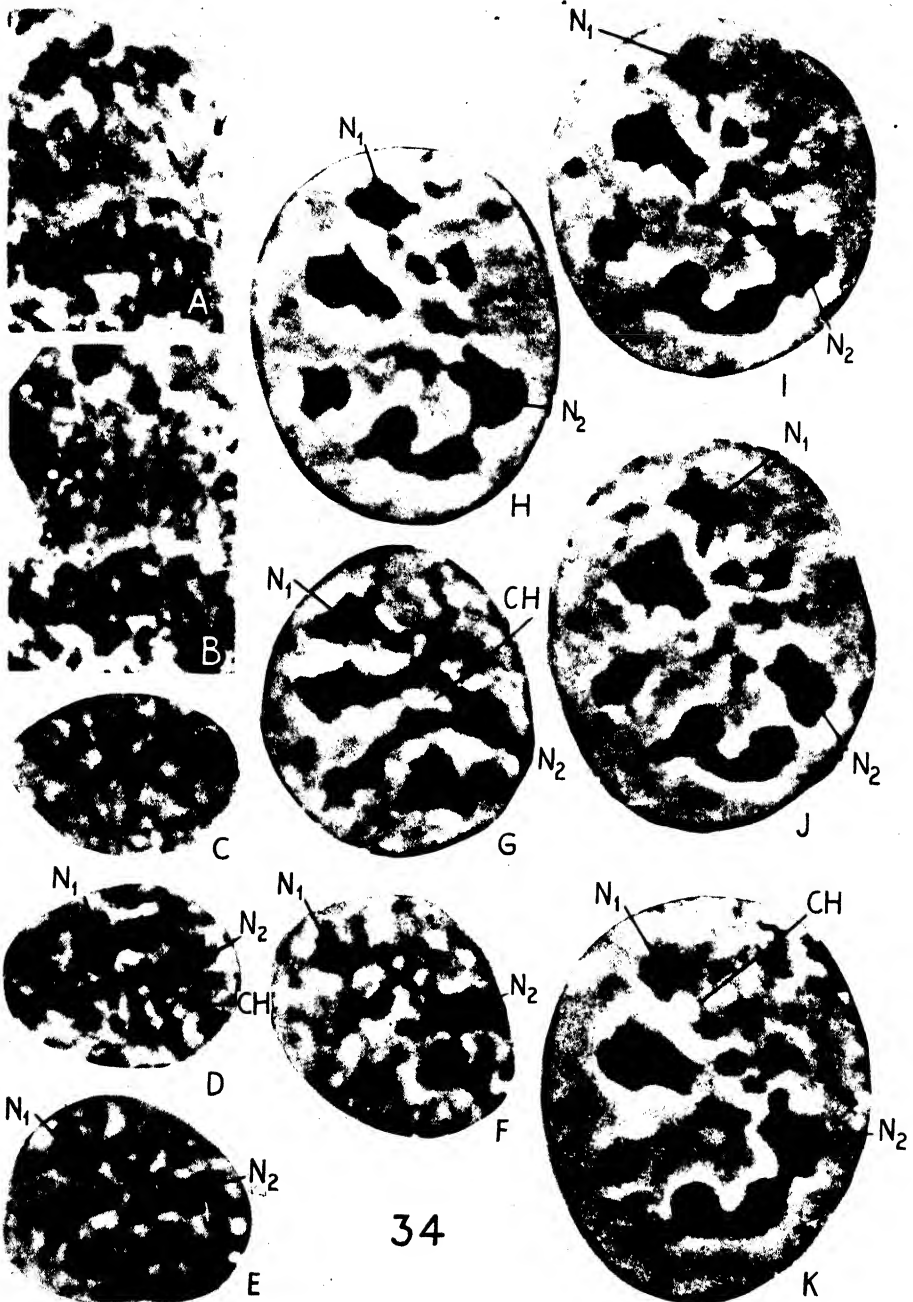


H. B. FELL AND A. F. HUGHES—PLATE IV

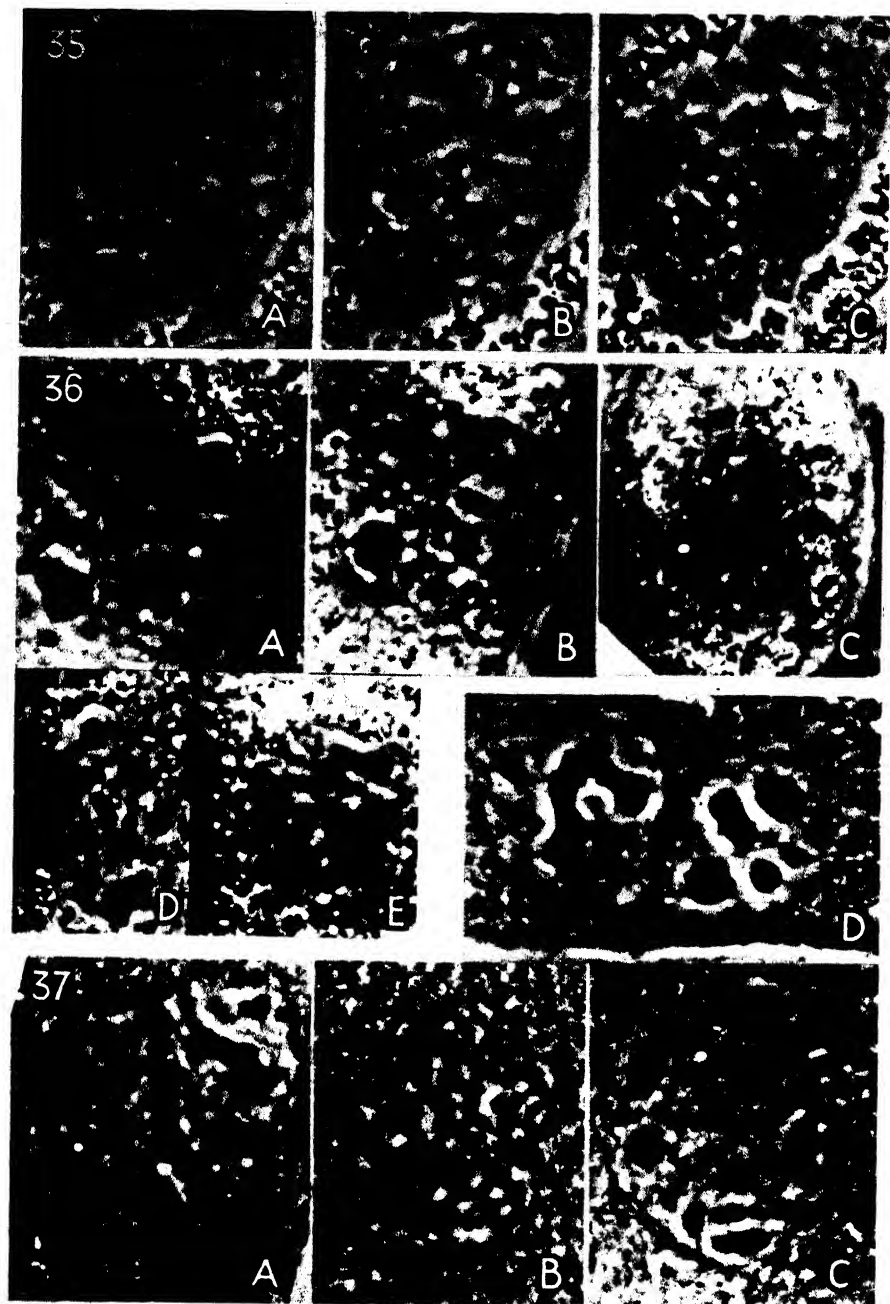


H. B. FELL AND A. F. HUGHES—PLATE V





34



The Distribution of Alkaline Phosphatase in Relation to Calcification in *Scyliorhinus canicula*

Development of the Endoskeleton

BY

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With one Plate

INTRODUCTION

IN teleosts and all higher vertebrates calcification is a preliminary to ossification. But in elasmobranchs calcified cartilage functionally replaces bone. The histology and biochemistry of elasmobranch cartilage have been reviewed by Weidenreich (1930). In spite of the vast literature on the subject, the relation of the calcium salts to the matrix is not fully understood and there is disagreement regarding the presence and amount of calcium phosphate. However, the following points seem established: calcification always starts at the periphery of a piece of cartilage, at a point remote from the cells. Amorphous granules are first formed which later coalesce into an irregular mosaic of crystalline plates. In *Scyliorhinus* the platelets cover the inner and outer surfaces of the vertebral arches and the skull and the total surfaces of the visceral skeleton. The pattern of calcification in the vertebral column of the higher elasmobranchs is so typical that it has been used as a basis of classification (Hasse, 1893). In view of these facts a study of the distribution of alkaline phosphatase in an elasmobranch seemed of interest. Here normal calcification can be studied without the complications of cartilage hypertrophy and resorption and the simultaneous formation of a new protein matrix which always accompany ossification.

Badansky, Bakwin, and Bakwin (1931) showed that alkaline phosphatase exists in elasmobranchs, but drew no correlation between phosphatase activity and calcification. The question was studied in greater detail by Roche and Bullinger (1939). These authors showed that the phosphatase present in the skeleton of both teleosts and elasmobranchs belongs to the Class 1A of Folley and Kay (1936); i.e. it is closely similar to that found in mammalian bones. By means of microchemical tests Roche and Bullinger showed that only the calcifying regions of elasmobranch skeletons contained the enzyme, adjacent soft portions being negative. These results as well as a study of phosphatase in relation to the growth of scales and teeth (Roche, Collet, and Mourgue, 1940) led the authors to conclude that in elasmobranchs, as in higher vertebrates, phosphatase is concerned with rapid calcification.

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No histochemical study of the fish skeleton seems to have been undertaken, nor has phosphatase been studied during the embryonic development of lower vertebrates. It therefore seemed desirable to undertake a systematic study of the histological and cytological distribution of alkaline phosphatase in developing teleost (Lorch, 1949) and elasmobranch embryos. Only the endoskeleton is considered here.

METHODS

Twenty egg-cases and five hatched specimens of *Scyliorhinus canicula* were obtained living from the Marine Biological Laboratory, Plymouth. After removal of egg-case and yolk-sac the specimens were anaesthetized in 10 per cent. ethyl alcohol, measured, and placed into 80 per cent. ethyl alcohol in distilled water. When sufficiently hardened they were divided into a number of blocks according to the size of the fish and fixation was continued up to 24 hours. It was found that when 80 per cent. alcohol in *sea* water was used as fixative, considerable destruction of phosphatase occurred. The reason for this is not clear. The heads of three of the hatched specimens were split longitudinally, one half being decalcified by my method (Lorch, 1947*b*) and the other cut undecalcified. It was found that 4 hours' decalcification (4 changes of buffer at 10° C. and pH 4.7) was adequate, but times up to 4 days did not result in an appreciable loss of enzyme. Reactivation was carried out at room temperature for 24 hours.

All specimens were dehydrated in alcohol, cleared in cedarwood oil or benzene, and embedded in paraffin (m.p. 56° C.). Serial sections (8 μ) were mounted without albumen. For the visualization of alkaline phosphatase Gomori's (1939) method as modified by Danielli (1946) was mainly employed. Incubation times were varied from 1 to 15 hours at 28° C. Some sections were treated by the diazo-method of Menten, Junge, and Green (1944), by means of which phosphatase can be visualized independently from calcium salts. However, only sites of very great enzyme activity can be demonstrated (Lorch, 1947*a*). The two-colour method involving the use of gallamine blue (Lorch, 1947*b*) was used to demonstrate calcium salts and phosphatase in the same section. Calcium deposits were also visualized by von Kossa's silver nitrate method, by staining the section for 5–15 mins. in a saturated neutral solution of gallamine blue (Stock, 1949), and by bulk alizarin red S staining. The usual histological stains were employed to elucidate general structure. A combination of methyl green, which stains cartilage metachromatically, and van Gieson's stain was found useful.

RESULTS

The description of phosphatase distribution is based on a study of 13 specimens which may conveniently be divided into 6 stages (see opposite). Purely anatomical features will not be described; reference should be made to de Beer (1931, 1937).

Stage	Length (mm.)	No. of specimens
1	18-20	2
2	32-35	2
3	47	1
4	58	1
5	75	2
6	96-115	5

1. Cartilage of the Skull

At the earliest stages examined (18-20 mm.) no cartilage is present. The first signs of precartilaginous tissue appear in the region of the future parachordals as condensation of mesenchymal cells. No phosphatase was detected in this tissue, nor in the developing cartilages of the 32-35-mm. specimens. At Stage 3 (47 mm.) the main features of the adult skull are established. The growing regions and the more recently formed cartilages are still negative for phosphatase, but a positive reaction is now given by the chondrocytes of some older cartilages. Centrally placed cells are only faintly positive but the flattened chondrocytes at the periphery stain strongly. Perichondral fibroblasts and fibres display phosphatase activity in most cases but never at the growing zones. In the parachordals, the oldest cartilages of the skull, the cells stain strongly and traces of phosphatase occur also in the matrix. Meckel's cartilage of the lower jaw may be described as typical of this stage: its anterior tip is just distinguishable from the connective tissue. It has no phosphatase activity. Distally, Meckel's cartilage is better defined and has a distinct positively staining perichondrium on the dorsal and ventral surface. Laterally the cartilage merges into connective tissue. Pl. I, fig. 1, shows the distribution of phosphatase in two jaw cartilages of a 47-mm. specimen. In the 58-mm. embryo the anterior parts of Meckel's cartilage are partly surrounded by positively staining perichondrium, leaving the central (growing) surfaces free. But in the mid-orbital region the strongly staining perichondrium forms a complete ring round the cartilage and patches of strongly positive chondrocytes make their appearance near the ventral edge. The matrix is also slightly positive here. Similar patches are seen in the pterygoquadrate and ceratohyal. Apart from these occasional well-localized patches the cartilage matrix is negative at this stage, but nuclear phosphatase occurs in some of the flattened peripheral chondrocytes and in the perichondral fibroblasts.

At Stage 5 a new phenomenon makes its appearance: the jaw cartilages, parachordals, and most of the brain case of the 75-mm. and older specimens display phosphatase activity in a well-defined pattern. The cartilage may be divided into three zones (Text-fig. 1). Immediately below the perichondrium the 2-3 rows of flattened cells continue to display phosphatase activity and the matrix may give a moderately strong reaction (Zone 1). This region shows less affinity for basic dyes than the rest of the cartilage matrix. Passing inwards the cells become more rounded and their phosphatase activity increases

sharply. Pericellular 'capsules' of intensely positive matrix are frequent. The matrix between the cells also stains strongly (Zone 2). Centrally to this layer of cells the cartilage abruptly loses its phosphatase activity, although it does not appear in any way different when ordinary staining procedures are used (Zone 3). The zones are best distinguished after relatively short incubation times and it becomes clear that the maximum phosphatase activity is found in Zone 2. When the growing region of a cartilage is examined it is seen that phosphatase appears in the following order: first in the perichondrium as soon as this is clearly defined, secondly in 'Zone 1' (flattened cells), and finally in the cells and matrix of Zone 2 where the reaction is most intense.

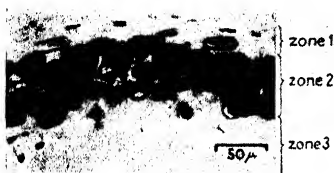


FIG. 1

TEXT-FIG. 1. From a decalcified transverse section through the pterygoquadrate of a 110-mm. specimen, showing the distribution of phosphatase at the periphery of the cartilage. Incubation time, 5 hours.



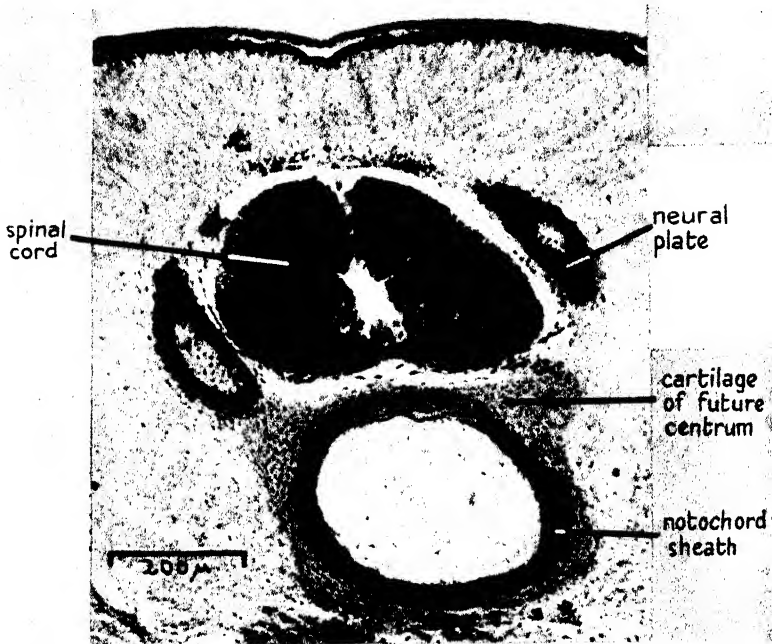
FIG. 2

TEXT-FIG. 2. Calcified platelets at the periphery of the parachordal cartilage of a 115-mm. specimen. Stained with gallamine blue and counterstained with safranin. Note position of platelets corresponds to 'Zone' 2 in Text-fig. 1.

As growth proceeds Zone 2 increases somewhat in thickness, but the negative core of cartilage (Zone 3) persists even in adult dogfish. Pl. I, fig. 2, shows the appearance of Meckel's cartilage and the pterygoquadrate at Stage 5.

In the skulls of embryos up to Stage 6, there was no sign of calcification detectable by histochemical methods. But in the hatched specimens (96–115 mm.) calcified platelets are seen at the periphery of some of the cartilages (Text-fig. 2). With regard to the distribution of phosphatase in these specimens there is no qualitative change in the pattern just described. However, Zone 2 is now present in a greater number of cartilages and has increased in thickness in places where it was previously found. The reaction is even more intense and can be detected after very short incubation times (1 hour). It is of interest to note that the calcified platelets are always located within Zone 2. The phosphatase reaction is more extensive than the region of calcification, but the reverse was never the case. The perichondrium, previously positive, now appears negative. Some of the smallest branchial cartilages are still devoid of phosphatase, whereas others show a patchy distribution of Zone 2. Meckel's cartilage, the pterygoquadrate, and the ceratohyal have broad, intense bands of phosphatase activity, and show most extensive peripheral calcification. In the anterior region of the parachordals extracellular phosphatase is present only near the ventral surface, whereas more distally ventral and dorsal positive bands are seen. Calcification is strictly correlated with this phos-

phatase distribution. Decalcified sections treated with haematoxylin and eosin or van Gieson's stain do not show any change in the matrix in the region of calcification, such as takes place in the calcifying cartilage of mammals, nor is there any hypertrophy of the phosphatase-containing cells similar to that seen during cartilage-bone formation in the trout (Lorch, 1949). In



TEXT-FIG. 3. Transverse section through the vertebral column of a 47-mm. specimen. Phosphatase shown black (Gomori method). There is no calcification. Incubation time, 4 hours.

cartilages of an adult dogfish examined, essentially the same distribution of phosphatase was found as in the calcified cartilages of the recently hatched specimens.

2. The Vertebral Column

Only the trunk region will be described. Up to the 35-mm. stage no phosphatase was detected in the notochord or in the rudiments of the vertebral cartilages. However, at Stage 3 (47 mm.) intense, well-localized areas of phosphatase activity are found. The matrix at the periphery of the neural arch cartilages is strongly positive as well as the chondrocytes both at the periphery and, to a lesser degree, in the centre (Text-fig. 3). Only the cartilage at the junction of neural plate and notochord sheath is entirely free from phosphatase. The intercalary tissue and the perichondrium are negative. Zones of phosphatase activity display an affinity for acid dyes: the periphery

of the neural arches stains pink with eosin or acid fuchsin and red with Heidenhain's Azan.

The spongy tissue of the notochord displays no phosphatase activity at this and subsequent stages, but the fibrous sheath foreshadows the future double-cone pattern of calcification in the vertebral centra by the distribution of phosphatase in the nuclei of spindle-shaped concentrically arranged cells. There is as yet no calcification in the vertebral column.

At Stage 4 (58 mm.) the pattern of phosphatase distribution becomes more sharply defined and the reaction more intense (Pl. I, fig. 3). The strongest reaction (very marked after 20 minutes' incubation) is given by the cells and fibres of the notochord sheath but only in the area of future calcification. The intervening tissue is negative, although as yet no histological differentiation can be seen within the future centrum. In the neural plates the cells and matrix are now positive throughout the cartilage, but short incubation times (1 hour) and application of the diazo-method show that the most intense reaction occurs in the matrix at the periphery (Text-fig. 4). The first signs of the calcification are now seen in the matrix just below the peripheral layer of flattened cells which is very narrow in the vertebral cartilages (Pl. I, fig. 4). No platelets of calcium salts similar to those in the skull have been noted. Sections treated by von Kossa's silver nitrate method show a granular deposit (Text-fig. 5). It is again noted that the phosphatase reaction is more widespread than the zone of calcification, but deposits of calcium salts do not occur in negative regions.

In the 74-mm. embryo calcification of the neural plates has progressed considerably: only the central core of the cartilage is now free from calcified deposits. There is a tendency for the granules to fuse and form a homogeneous calcified zone (Pl. I, fig. 6). In the specimens above 95 mm. granular deposits are no longer seen. The phosphatase activity of the neural plates becomes less as calcification increases: at Stage 5 some positive chondrocyte nuclei are encountered within the most recently calcified zone, but heavily calcified regions are quite negative (Pl. I, fig. 5). At the junction between the calcified outer layer and the uncalcified core of cartilage the matrix continues to display phosphatase activity. Hence it appears that the phosphatase positive zone is progressively pushed inwards by the advance of calcification. There is some overlap when phosphatase and calcium salts occur in the same region.

The matrix of the calcified cartilage becomes increasingly more acidophil, while uncalcified cartilages such as the neural spines show the usual affinity for basic dyes. In the hatched specimens the strongly calcified periphery of the neural plates have irregularly shaped lacunae which contain the chondrocytes. The latter also show various shapes and do not resemble the spherical chondrocytes of the non-calcified cartilage which have basophil granules in the cytoplasm. In the transition zone (phosphatase positive) some chondrocytes have a strongly acidophil cytoplasm devoid of granules, while others are still granular.

The notochord sheath first shows a zone of calcification in the 75-mm. embryo, i.e. somewhat later than the neural plates. The pattern already established in the 47-mm. specimen by the phosphatase distribution is main-



FIG. 4

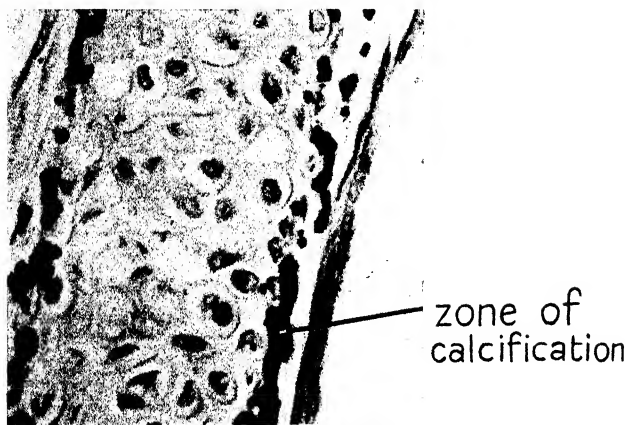


FIG. 5

TEXT-FIGS. 4 and 5. From the neural plate of a 58-mm. specimen. In Fig. 4 phosphatase is shown as $\text{Ca} \alpha$ naphthyl phosphate (modified method of Menten, Junge, and Green, 1944). Fig. 5 shows the distribution of calcium salts (von Kossa's nitrate method).

tained. The calcium deposits occur in the middle zone of the fibrous sheath, where a layer 2-3 cells thick stains heavily with silver nitrate (Pl. I, fig. 6). The elongated cells, surrounded by calcium salts, are still well defined and display some phosphatase activity. But in the oldest specimens, where the calcified zone is much thicker, the cells are negative.

The inner zone of the fibrous sheath displays strong phosphatase activity in all 74–115-mm. specimens, but the outer zone becomes progressively less positive. In the intervertebral regions there is no calcification and no phosphatase. No secondary calcification of the outer zone of the notochord sheath was noted. This is said to occur (Hasse, 1893).

The relation between phosphatase and calcification in the skeleton of the series of dogfish embryos examined is summarized in Table 1.

DISCUSSION

The following points emerge clearly from this study: phosphatase is absent from the cartilages of the young embryos and appears first in the chondrocyte nuclei and in the perichondrium. At a stage just prior to calcification the enzyme is also detected in the cartilage matrix. The maximum extracellular phosphatase activity occurs during the first stages of calcification. The phosphatase positive zone then retreats in front of the wave of calcification. This is most clearly seen in the neural plates where calcification has reached the most advanced state. In the skull the stage of secondary reduction of phosphatase activity has only just been reached in the hatched specimens. It seems, therefore, that in dogfish, as in teleosts and mammals, phosphatase is an essential pre-requisite for calcification. Moreover, it is noted that calcification only occurs in regions where *extracellular* phosphatase is present, but there is a lag between the appearance of the enzyme in the cartilage matrix (or notochord sheath) and the onset of detectable calcification. It is interesting to note in this connexion that no extracellular phosphatase could be detected in the cartilage and notochord sheath of an adult lamprey (*Petromyzon fluviatilis*), although the peripheral chondrocytes gave a positive reaction as in young dogfish embryos. The skeleton of cyclostomes does not calcify. To save space no description of the distribution of phosphatase in tissues other than the skeleton has been given, but it must be understood that the enzyme is present in tissues other than those here described. Taking the embryo as a whole the conclusions reached with developing trout (Lorch, 1949) are confirmed: nuclear phosphatase shows no relation to calcification, whereas extracellular phosphatase is only found in connexion with calcification or fibre formation.

There is one notable difference between calcification in the dogfish and cartilage bone formation in the trout: in the latter the chondrocytes enlarge, the matrix becomes basophil and a perichondral layer of pre-osseous substance is formed prior to calcification. However, in *Scyliorhinus* the calcium salts are precipitated within the cartilage and neither the matrix nor the cells show any preliminary change other than the phosphatase distribution. (The change in staining reaction described by Weidenreich [1930] and noted in the vertebrae of the older specimens is secondary to calcification.)

Whereas one cannot exclude the possibility of phosphatase playing a part in the formation of the pre-osseous matrix in teleosts and mammals, such a role is unlikely in elasmobranchs. The simplest hypothesis is therefore that

TABLE I

SKULL CARTILAGES

VERTEBRAL COLUMN

STAGE	PHOSPHATASE IN CHONDROCYTES		MATRIX	CALCIFICATION OF MATRIX	NEURAL PLATES		NOTOCHORD SHEATH		REMARKS
	PERICHONDRIUM				PHOSPHATASE	CALCIFICATION	PHOSPHATASE	CALCIFICATION	
1									NO CARTILAGE; PRECARTILAGE NEGATIVE.
2	—	—	—	—	—	—	—	—	
3	+	—	—	—	+	—	+	—	
4	+	+	+	—	++	+	++	—	
5	+	++	+++	—	+++	++	+++	+	
6	+	+++	+++	+	+	+++	+++	+++	
ADULT	—	+	++	+++					VERTEBRAE NOT EXAMINED

phosphatase is here solely concerned with the precipitation of calcium phosphate.

ACKNOWLEDGEMENTS

I should like to thank Professor J. F. Danielli for reading the manuscript and Professor Samson Wright for providing facilities for this work, which was financed by a grant from the Medical Research Council.

SUMMARY

1. A histochemical study has been made of alkaline phosphatase in the endoskeleton of dogfish embryos of 18–115 mm.

2. The distribution of phosphatase is compared with that of insoluble calcium salts.

3. Phosphatase was first noted in the chondrocytes and perichondrium at 47 mm. and in the cartilage matrix at 58 mm.

4. Calcification occurred first in the neural plates at 58 mm. and in the skull at 74 mm. No calcification was observed in zones devoid of extracellular phosphatase.

5. As the intensity of calcification increased the amount of phosphatase tended to drop.

6. It is concluded that in elasmobranchs, as in higher animals, extracellular phosphatase is an essential precursor of calcification.

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DESCRIPTION OF PLATE I

FIG. 1. Transverse section through the jaws of a 47-mm. specimen. Sites of phosphatase activity black (Gomori method). Incubation time, 3 hours. There was no calcification.

FIG. 2. Transverse section through the jaws of a 75-mm. specimen. Sites of phosphatase activity black (Gomori method). There was no calcification. Note the patchy distribution of phosphatase in the cartilage matrix. Incubation time, 3 hours.

FIGS. 3 and 4. Serial transverse sections through the vertebral column of a 58-mm. specimen. Fig. 3 incubated 6 hours for phosphatase visualization (Gomori method). Fig. 4, unincubated control showing zone of calcification.

FIGS. 5 and 6. Serial transverse sections through the vertebral column of a 75-mm. specimen. Fig. 5 shows the distribution of phosphatase in a decalcified section (Gomori method, 3 hours' incubation); Fig. 6 shows the extent of calcification (von Kossa's method). Note the regression of phosphatase from heavily calcified zones.



FIG. 1



FIG. 2

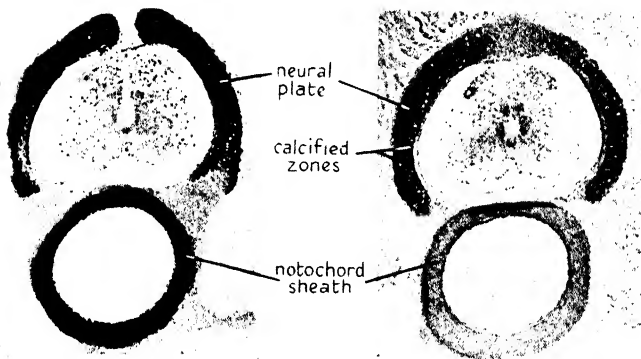


FIG. 3

FIG. 4

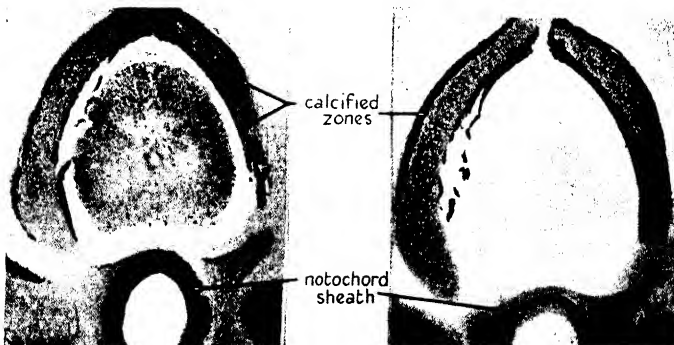


FIG. 5

FIG. 6

Alkaline Phosphatases and the Cycle of Nucleic Acids in the Gonads of Some Isopod Crustaceans

BY

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With one Plate

INTRODUCTION

MUCH work has been done with a view to localizing alkaline phosphatases in various tissues by means of the technique of Takamatsu (1939) and Gomori (1939). The advantage of this technique is that it is based on a chemical reaction and thus highly specific, at least for a certain group of enzymes. Furthermore, critical observations, mainly by Danielli (1946) and Lison (1948), indicate that this technique not only shows great specificity but also makes it possible to localize fairly exactly the sphere of the enzymatic action; one can assume with a reasonable degree of safety that the areas which appear positive exercise *in vivo* enzymatic activity.

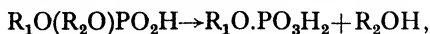
Recent work by Krugelis (1947) on proliferating tissues of certain marine invertebrates has shown a varying distribution of alkaline phosphatases in the various stages of development. The author has come to the conclusion that their distribution does not completely coincide with that of the ribonucleic acid in the cytoplasm.

The present work was carried out on the gonads of some Isopod Crustaceans (*Asellus aquaticus*, *Meinertia parallela*, and *Anilocra physodes*) with a view to controlling the latter point and finding out what relation exists between the phosphatases and ribonucleic (RNA) and thymonucleic (DNA) acid. As they are phosphorus compounds it is likely that phosphatases are concerned in their metabolism.

So far, little is known either of the chemical nature of phosphatases or of their classification. All that can be said is that they break the bonds between phosphoric acid and various organic radicles.

Phosphatases have been classified by Lison (1948) into monoesterases and diesterases. Monoesterases act upon compounds of the type $R_1O.PO_3H_2$, whereas diesterases act upon compounds of the type $R_1O(R_2O)PO_2H$. The technique of Takamatsu and Gomori reveals the presence of alkaline phosphatases by splitting off phosphate from glycerophosphate: the phosphate is precipitated by the calcium present in the medium, and the precipitate, which may be visualized by several methods, reveals the site of the enzyme.

In the case of the diesterases it is doubtful whether a precipitable compound would be formed as a result of enzyme action: the action would normally be



and both the compounds $R_1O.PO_3H_2$ and R_2OH will normally be as soluble as, if not more soluble than, the initial compound. But one of the two products, $R_1O.PO_3H_2$, is a substrate for monoesterases: consequently in tissues which are rich in alkaline phosphatase the products of the activity of diphosphatase may be subjected to the action of monoesterase before they have diffused far. Thus, if a tissue rich in monoesterase is exposed to the substrate for diesterase, the site of diesterase may be indicated in a rough way by the product of the consecutive reactions diester \rightarrow monoester \rightarrow free phosphate. The localization of diesterase so obtained must necessarily be lacking in precision, and of uneven value.

Here care has been taken to distinguish these two types of enzymes, mono- and diphosphatases. As substrate for monophosphatases sodium- β glycerophosphate, and for diphosphatases RNA and DNA were used.

A preliminary note of the results presented in this paper was published in *Experientia* (Montalenti and de Nicola, 1948b).

MATERIAL

The material used was the gonads of *Asellus aq.* and of some Cymothoids (*Anilocra physodes* and *Meinertia parallela*). The stages investigated were: in spermatogenesis, from spermatogonia to spermatocytes, spermatids, and spermatozooids; in oogenesis, from oogonia to oocytes in diplotene and first stage of the formation of auxocytes, when the chromatin is in the so-called 'diffuse stage'.

TECHNIQUE

The technique used was Gomori's method with slight modifications as already suggested by other workers.

The best fixation obtained was with acetone at a temperature of $4^\circ C$. for 12–24 hours, followed by treatment with abs. alcohol for 1 hour. Then 10 minutes in xylene, followed by embedding in paraffin (changed twice) at $52^\circ C$. for a total length of 15 minutes. The pieces used were very small. They were immediately sectioned at 5μ and the paraffin was removed within 12 hours. They were then put in an incubator at $38^\circ C$. Some sections were kept in the refrigerator for a few days and did not lose their enzymatic activity. The incubation solutions were, as recommended by Gomori, at pH 9.5, only the substrates being different. These consisted of sodium- β glycerophosphate for monophosphatases, polymerized DNA (containing 1.2 per cent. protein), and RNA.

The duration of incubation varied, according to the kind of material, from 8 to a maximum of 72 hours, for the nucleic acids. Due consideration was taken of recent objections to Gomori's technique. It is assumed, however, that the technique used, i.e. fixation at a low temperature, rapid embedding, and rapid paraffin removal, should reduce to a minimum the unavoidable partial inactivation of the enzyme during the histological preparation.

Control sections were kept at $90^\circ C$. for 15 minutes before incubation, or

in $\text{Hg}(\text{NO}_3)_2$ for an hour at normal temperature. This was observed to produce inactivation of the alkaline phosphatases (Montalenti and de Nicola, 1948).

By means of this technique the cellular structure remains unaltered and phosphatases are entirely destroyed. Furthermore, other control sections were kept in Gomori's solution without substrate for the same length of time. They always remained unstained. In all slides the staining was equally distributed and of constant intensity, which proves that there are no perceptible variations due to technical errors.

OBSERVATIONS

Spermatogenesis and Polyploid Nuclei of Follicular Cells

Experiments were carried out during various stages of the spermatogenesis of *Asellus aquaticus* and Cymothoids. The general result was that the phosphatases decrease during the development from spermatogonium to spermatocyte and to spermatid. They are completely absent in the latter with the exception of some traces along the external membrane and particularly at one point in it. Slightly positive results are obtained in the spermatozooids (Pl. I, fig. 2). This decreasing intensity of staining finds its counterpart in a similar behaviour towards ordinary nuclear stains.

The chromosomes are always positive for phosphatase (Pl. I, figs. 1 and 3). The nuclei of the follicular cells of the testis of *Asellus*, i.e. the giant polyploid nuclei, undergo a cycle of variations which may be related to that of the germ cells (Vitagliano, 1948). They give rise to RNA, which is put out in form of granules which pass between the germ cells. The relationship between the nucleic acids during this process was investigated (de Nicola, 1948) in order to ascertain what part the phosphatases play during the synthesis of RNA. Four stages of polyploid nuclei have been described with ordinary nuclear stains, corresponding to four stages of distribution of phosphatases:

Stage I: Nucleus with granular chromatin. One or two nucleoli present. Small quantity of RNA. In the cytoplasm it is almost completely absent. Monophosphatases and diphosphatases exist in nucleoli and in the chromatin granules, not in the cytoplasm.

Stage II: Nucleus with diffuse chromatin. Five or six nucleoli. Small granules of RNA in the cytoplasm. Monophosphatases only in the nucleoli. Diphosphatases only in two or three nucleoli. It must not be overlooked that RNA is present only in two or three of these nucleoli while all of them stain by iron haematoxylin.

Stage III: Nucleus much contracted and intensely Feulgen positive. Cytoplasm full of RNA. One or two nucleoli. During this stage the polyploid cells produce the maximum quantity of RNA granules. Great quantities of monophosphatases and diphosphatases in the nucleus.

As was pointed out in the publication of Vitagliano (1948), stages II and III occur twice during the spermatogenetic cycle before the onset of the fourth stage.

Stage IV: Nucleus less contracted than in the previous stage, chromatin in form of numerous Feulgen-positive granules. One or two nucleoli. Cytoplasm presents moderate quantity of RNA. Monophosphatases and diphosphatases are present in the granules of the chromatin and in the nucleoli.

In conclusion, the phosphatases exist only in the polyploid nuclei and in the nucleolus of the germ cells: they correspond in these two areas to the distribution of the nucleic acids. They are completely absent in the cytoplasm and the RNA granules which are expelled from the cytoplasm.

Thus phosphatases exist only where there is a synthesis (polyploid nuclei) or re-elaboration (nuclei of germ cells) of the nucleic acids. The cytoplasm, which plays no part in this process, is devoid of these enzymes. The granules, which are no longer in metabolism during their way from polyploid nuclei to germ nuclei, do not contain any phosphatase though they are rich in RNA.

The polyploid nuclei of the follicular cells of the testis of Cymothoids are also strongly positive (Pl. I, fig. 2), but I did not investigate whether stages similar to those mentioned above can be recognized in this material.

OOGENESIS

Monophosphatases

A progressive decrease of enzymes is observed in the course of the development from the oogonium in *Asellus aquaticus*: typical chromosome figures are stained by Gomori's reaction (Pl. I, fig. 3). The cytoplasm becomes considerably less positive during development. The same applies to the nucleus though the reaction is never totally negative.

Identical observations were made with Cymothoids where more detailed observations could be made, owing to the large size of the oocyte. One, or sometimes (especially in earlier stages) two nucleoli are observed which react with the same intensity in all stages (Pl. I, fig. 6). Chromocentres were also noted, consisting only of DNA showing a positive reaction to monophosphatases and diphosphatases of DNA.

In the remaining part of the nucleus phosphatases are distributed according to the chromosome structure typical for each species. The form of the bivalents at the beginning of the 'diffuse stage' shows, indeed, a marked and typical difference between *Meinertia parallela* and *Anilocra physodes*. In the former the bivalents form small blocks of irregular outline while in the latter they retain their elongated and filamentous shape. These structures as they appear by means of ordinary nuclear dyes correspond exactly to the structures obtained by Gomori's reaction (Pl. I, figs. 4 and 5).

In the oocyte at the maximum size observed, in which vitellogenesis is well advanced and the chromatin practically all diffuse, the phosphatase reaction is very weak (Pl. I, fig. 6). The quantity of phosphatases is considerable in the cytoplasm during the earlier stages. It decreases and almost disappears as vitellogenesis proceeds (Pl. I, fig. 6).

The small diploid nuclei of the follicle cells show a very intense reaction

for monophosphatases, and DNA and RNA diphosphatases. They do not present any cycle which could be compared with that of the polyploid nuclei of the testis.

Diphosphatases

As mentioned above, experiments were also carried out on alkaline diphosphatases using DNA and RNA as substrate.

The diphosphatases of RNA exist in the chromosome structure of the nucleus and—homogeneous and diffuse—in the cytoplasm of all the stages examined. They correspond to the distribution of RNA which is particularly abundant in the nucleus, as I have ascertained by means of the Unna-Pappenheim reaction.

The diphosphatases of DNA show a distribution corresponding to that of DNA in the nucleus; indeed, the characteristic blocks of chromatin appear in *Meinertia*. They also exist in the nucleoli and in the cytoplasm, but in neither of these do they correspond to the distribution of DNA which is completely absent.

Both diphosphatases exist in the nuclei of follicular cells.

DISCUSSION

Monophosphatases

The localization of alkaline monophosphatases closely follows the distribution of DNA and RNA. In the nucleus the structures blackened by Gomori's reaction are identical with these stained by the common nuclear dyes. The nucleolus is always strongly positive. This is not unexpected, because phosphatases, which are dephosphorylating enzymes, may well be present where any synthesis or degradation of phosphorus compounds such as nucleic acids occurs.

Therefore the cycle of phosphatases might be interpreted as simultaneous with the cycle of nucleic acids. In fact, spermatogonia and oogonia are particularly rich in phosphatases, possibly because an active synthesis of nucleic acids takes place in them during successive mitoses and meioses. When the spermatid stage is reached, the synthesis practically comes to its end, and the Feulgen reaction is pale.

The nuclei of oocytes during yolk formation also have ceased to metabolize DNA. The oocyte cytoplasm during the earlier stages is particularly rich in enzyme. This is probably necessary for RNA synthesis, which, in turn, is required for protein synthesis during the subsequent period of enormous increase in size. At the beginning of yolk formation RNA diminishes and eventually disappears entirely. Caspersson (1936-40), by means of ultraviolet absorption, has observed a diminution of RNA running parallel to the size increase, and this has been confirmed by Brachet (1944). The diminution has been confirmed in the material used in this work by means of the Unna-Pappenheim method. Monophosphatase also diminishes and finally disappears almost entirely.

Such a strict correlation of monophosphatase and nucleic acids only exists where the latter undergo synthesis or degradation. In the follicular cells of the *Asellus* testis, only the nucleus, when the synthesis occurs, is rich in enzyme. The cytoplasm, in which a considerable amount of RNA is stored which is not in active metabolism, is entirely devoid of phosphatases. The same is true for the granules which are expelled from the cell nuclei. In the germ cell nuclei, in which synthesis takes place, phosphatases are again to be found.

It may therefore be concluded that the strict connexion between phosphatases and nucleic acids distribution exists only where metabolism of nucleic acids occurs.

Another conclusion may be reached, which is in disagreement with Krugelis's statement. Krugelis (1942) has noticed in various tissues that DNA diphosphatase can be detected only by means of depolymerized DNA. This would be expected since free phosphoric groups are present only after depolymerization. On the contrary, in the present work, a positive Gomori's reaction has been obtained with highly polymerized DNA. This happens with very long incubation time (48 to 52 hours) while the usual time in this material is only 8 hours. Possible explanations are the following: (a) DNA spontaneously depolymerizes during the long incubation time; (b) a nuclease is present together with phosphatases; (c) diphosphatases have also a nucleasic action.

The first possibility may be excluded because incubation solutions previously kept at 38° for 72 hours still require a further 48 hours' incubation with the slides in order to give the reaction on them. Furthermore, the viscosity of incubation solutions kept at 38° for 48 hours was the same as in controls kept in the ice-box. Thus the degree of polymerization is not altered by temperature or alkalinity.

Diphosphatases

The distribution of diphosphatases is more difficult to explain. Their specificity in respect to the substrate has been demonstrated by Krugelis (1947), and is generally accepted; but this author has found a much more localized distribution than that resulting from the present observations. Krugelis has found DNA diphosphatases exclusively in the nucleus and nucleolus, and RNA phosphatase in cytoplasm and nucleolus only. On the contrary, in our material both diphosphatases showed the same distribution. Both DNA and RNA used were carefully purified, and therefore it is improbable that the results are due to impurities.

The distribution of RNA diphosphatases present in nucleus, nucleolus, and cytoplasm corresponds to RNA localization as verified by Unna-Pappenheim's staining method, but they do not follow exactly the RNA cycle. Unlike monophosphatases, they do not show a perceptible diminution in the various stages.

DNA diphosphatases are present in the nucleus on the chromosome structures. Thus they correspond to DNA localization, but they are found also

in the nucleolus and in the cytoplasm, when DNA is entirely absent. These data are admittedly hard to explain. Since the nucleolus is involved in heterochromatin transformation one may think that a transformation of DNA into RNA occurs when the chromosomes enter the 'diffuse stage', and that it contains the specific diphosphatases.

In the cytoplasm DNA diphosphatases remain along the exterior border of the nuclear membrane even when the yolk formation has started. They might play a role in the transformation of DNA into RNA which has to be put out from nucleus into the cytoplasm. In fact, DNA diminution in the nucleus somewhat precedes that of RNA in the cytoplasm: when chromatin has already almost disappeared, the cytoplasm is still slightly positive to RNA reaction. The persistence of diphosphatases in nucleus and cytoplasm might perhaps mean that they represent a storage of enzymes of the cell, being independent within certain limits of the nucleic acid cycles.

As to the interpretation of the distribution of diphosphatases, some objections must be raised. First, it must be recalled that in order to demonstrate the diphosphatases by Gomori's method, the simultaneous presence of monophosphatases is required, which must be present to liberate phosphoric acid, which subsequently precipitates as an insoluble phosphate. Thus the possible existence of diphosphatases only cannot be demonstrated by this method. This means that a negative diphosphatase reaction cannot necessarily be taken as a proof of their absence, but may be due to lack of monophosphatases in the proper place.

Furthermore, since the demonstration of diphosphatases depends on monophosphatase localization, it may happen that the substrate, degraded by diphosphatases, shifts towards regions where monophosphatases are present, if these are located nearby. In this way the final reaction would take place where monophosphatases are present and the method would show the localization of the latter instead of diphosphatases.

Undoubtedly this hypothesis has some value. However, it does not appear to apply to the material which is the subject of this paper, for the following reasons: as already stated, polyploid nuclei of the follicular cells of *Asellus* testis at a given stage have six nucleoli, three of which show the monophosphatase reaction only, and three show mono- and diphosphatase reactions. If the above hypothesis were correct, the same reaction would be expected in all of them by using only diphosphatase substrate, owing to the propinquity of nucleoli. That is to say that the mononucleotides set free by diphosphatases belonging to the three first nucleoli would move on to the other three containing only monophosphatases and produce the reaction there.

This does not happen; therefore the diffusion hypothesis must be discarded, at least in this case.

I am deeply indebted to Prof. G. Montalenti for his constant advice and encouragement. I also want to express my deep gratitude to Prof. J. Danielli for reading the manuscript and for helpful advice and criticism.

The marine material was supplied by the Zoological Station of Naples, to which I express my gratitude. DNA and RNA acids, prepared in the laboratories of Prof. Caspersson, were kindly supplied by Prof. J. Runnström and I take this opportunity of thanking him warmly for his kindness.

SUMMARY

1. The distribution of alkaline phosphatases and their relation to nucleic acids have been investigated by means of Gomori's technique in the gonads of Isopod Crustaceans (*Asellus aquaticus* and some Cymothoids).

2. Alkaline monophosphatases are distributed over the Feulgen-positive structures, in nucleolus, and in the cytoplasm of young oocytes. They disappear from every place, except nucleoli, during yolk formation. The close coincidence of their localization with that of nucleic acids indicates that they are involved in the metabolism of the latter. The cytoplasmic localization is probably connected with RNA distribution only where this is being actively metabolized.

3. RNA diphosphatases have a similar distribution; they are present where the acid is present, but they do not follow so closely its cycle. Contrary to monophosphatases, they do not disappear gradually from the cytoplasm of the oocyte, where RNA does so.

4. DNA diphosphatases are partially independent from DNA, because they are present also in some Feulgen-negative structures (cytoplasm and nucleolus). As the RNA diphosphatases, they do not disappear gradually during vitellogenesis.

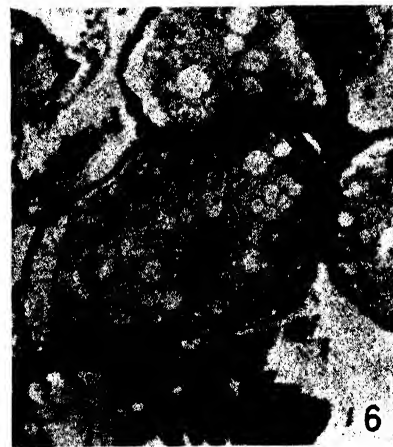
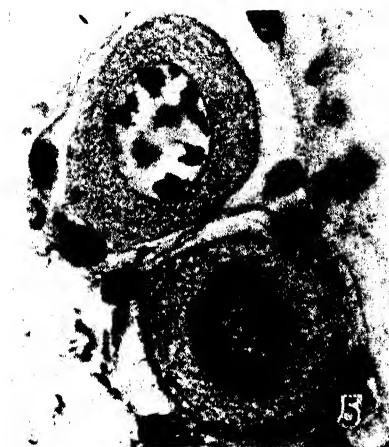
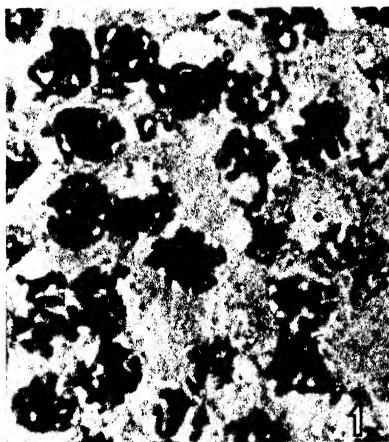
5. Diphosphatases show also a depolymerizing action on DNA. It seems more likely that this fact is due to a nucleasic action intrinsic in diphosphatases than to an association of these enzymes with a nuclease.

6. The interpretation of diphosphatases as a storage of phosphorylizing enzymes in the cell is discussed.

7. The possibility that Gomori's reaction of diphosphatases fails to show their exact localization in the cell is considered, and, as far as the present material is concerned, discarded.

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DESCRIPTION OF PLATE I

FIG. 1. *Meinertia*. Diphosphatases of polymerized DNA: spermatocytes. $\times 1030$.

FIG. 2. *Meinertia*. Polyploid nucleus of follicular cell and spermatozoa. Diphosphatases of polymerized DNA. $\times 1030$.

FIG. 3. *Asellus*. Monophosphatases: oocyte. Chromosomes and nucleolus strongly positive. $\times 1030$.

FIG. 4. *Meinertia*. Monophosphatases: oogonia and young oocytes. The characteristic blocks of chromatin (bivalents) and cytoplasm strongly positive. $\times 1030$.

FIG. 5. *Meinertia*. Monophosphatases: young oocytes. Bivalents and cytoplasm strongly positive. $\times 1030$.

FIG. 6. *Anilocra*. Monophosphatases: oocyte at the beginning of vitellogenesis. Chromatin in 'diffuse stage'. $\times 285$.

Cytochemical Studies on the Embryonic Development of *Drosophila melanogaster*

I. Protein Sulphydryl Groups and Nucleic Acids

BY

T. YAO

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IN experimental embryology the egg of *Drosophila* is known as a type of so-called mosaic egg as established by the experimental work of Geigy (1931a and b) and others. It is also a well-established fact that mosaic eggs sometimes show differential distribution of particular chemical substances either before fertilization or in very early embryonic stages. Thus the application of cytochemical methods to embryological studies has already yielded results of considerable interest (cf. Needham, 1942; Brachet, 1947a). On the other hand, in spite of the fact that *Drosophila* has long been a favourite material for genetic research, comparatively little work has been done on its embryology. It is for these reasons that I considered it advisable to make some cytochemical observations on the *Drosophila* egg. The present paper deals chiefly with the distribution of the protein sulphydryl groups and ribonucleic acid, since these substances are known to have direct bearing on axiation in vertebrate embryos (Brachet, 1947a).

MATERIAL AND METHODS

Wild-type Oregon S stock of *Drosophila melanogaster* Meig. was used and cultured by the usual technique at $25 \pm 0.2^\circ \text{C}$. Each cytochemical test has been carried out both on developing oocytes and on embryos of different stages. Oocytes in various developmental stages were obtained from 1-day-old females which contain practically all successive stages, whereas some of the intermediate stages are missing if females older than 1 day are used. For the collection of fertilized eggs, virgin females were kept separate from the males for 2 days. They were then mated and the males removed after 1 hour. Thirty to fifty such females were put into a single empty milk bottle and supplied with yeast paste. After another hour or two eggs were collected on a simple medium (2 per cent. agar containing 2 per cent. ethyl alcohol and 1 per cent. acetic acid) on a glass plate. The flies deposited eggs in quick succession under these conditions. After discarding the first two or three lots (which may contain some eggs with advanced embryos), eggs were collected at 10-minute intervals, timed, and incubated. The embryos for study were first de-chorionated free-hand as described by Poulson (1937). It was found that a small piece [Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

of the agar medium, after partially drying out, effectively sticks the embryo to the glass plate preparatory to de-chorionation. The embryos were then carefully punctured in the fixative with a very fine tungsten needle. A formal-alcohol-acetic acid mixture (Huettner, 1923) was used as fixative. Gonads and embryos were embedded in the usual way and sectioned at 10μ .

For the detection of protein sulphydryl groups (fixed —SH groups hereafter), the method devised by Chèvremont and Frédéric (1943) was employed. Control sections were treated with saturated mercuric chloride for 1 hour to block the —SH groups. Ribonucleic acid was demonstrated by the method of Brachet (1942): crude ribonuclease was isolated from calf pancreas: sections were incubated in a solution of this enzyme for 1–2 hours at 37.5°C .: control sections were incubated for the same period in distilled water. Deoxyribonucleic acid was studied by the standard Feulgen technique (Stowell, 1945).

In order to get some information about mature sperm, some cytochemical tests were repeated on *Drosophila* testes taken from 1-day-old male flies.

MORPHOLOGICAL OBSERVATIONS ON OOCYTE DEVELOPMENT

Since the development of the *Drosophila* oocyte has not been adequately described, it must first be summarized here. The ovarian history can be divided arbitrarily into eight stages, based on the size of the oocyte and other morphological characters of the nurse cells and follicular epithelial cells. These stages are shown in Text-fig. 1 and a brief description of them is given below (the median cross-sectional area (A) of a drawing of the oocyte was measured by a planimeter under a linear magnification of 160; the diameter (d) of the largest nurse-cell nucleus was measured by an ocular micrometer):

(1) $A = 25\text{ mm.}^2$, $d = 15\mu$. The oocyte can be distinguished from the nurse cells by its smaller-sized nucleus and less basiphilic cytoplasm.

(2) $A = 160\text{ mm.}^2$, $d = 20\mu$. The follicle elongates and the follicular cells become flattened out at the nurse-cell end: the nurse-cell cytoplasm becomes strongly basiphilic.

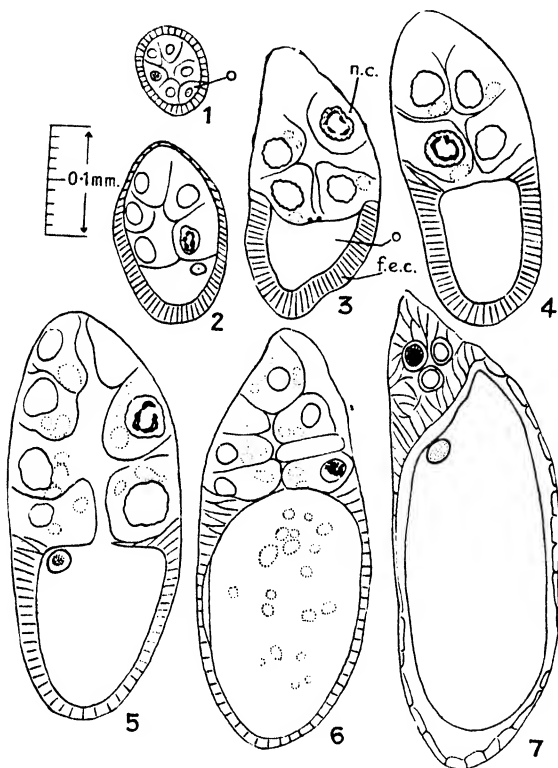
(3) $A = 310\text{ mm.}^2$, $d = 27\mu$. Deposition of yolk granules starts at the peripheral region of the oocyte. Follicular cells concentrate around the egg: a very thin envelope consisting of a few squamous cells is all that encircles the set of nurse cells (which probably permits an easy access of the raw materials from the body fluid). The cytoplasm of both nurse cells and follicular cells is strongly basiphilic. Intracellular vacuoles appear in the nurse cells.

(4) $A = 560\text{ mm.}^2$, $d = 40\mu$. Secretory activities of the nurse cells and follicular cells are in full swing. The nurse cells are highly vacuolated and their cytoplasmic contents start to pour into the egg. Not infrequently, whole nurse-cell nuclei can be found inside the ooplasm.

(5) $A = 1,080\text{ mm.}^2$, $d = 40\text{--}42\mu$. The general features of this stage are similar to those of the preceding stage. However, the physiological activity of the nurse cells has probably passed its peak as evidenced by the decline of

cytoplasmic basiphily: their cell walls are often found broken in the sectioned preparations. The follicular cells show no signs of degeneration.

(6) $A = 2,030 \text{ mm.}^2$ The nurse cells show a steady decrease of cytoplasmic volume and basiphily. Their nuclei became pycnotic, those adjoining the oocyte often being the first affected. A great number of vacuoles can be seen



TEXT-FIG. 1. Camera lucida sketches of the different stages of the oocyte development of *Drosophila melanogaster*. From iron haematoxylin preparations. *f.e.c.*, follicular epithelial cell; *n.c.*, nurse cell; *o.*, oocyte. Descriptions in the text.

in the oocyte cytoplasm: these are derived from the nurse cells and are observable under living conditions.

(7) $A = 2,600 \text{ mm.}^2$ The cytoplasmic contents of the fifteen nurse cells are completely absorbed by the oocyte. The follicular cells become flattened, but their cytoplasm is still moderately basiphilic. The whole egg cortex is covered by a thin, homogeneous, yellowish, and refractile structure—the vitelline membrane.

(8) This stage corresponds to a mature oocyte and is not shown in the figure. The follicular cells degenerate and the chorion with filaments is well formed.

No pycnotic nurse-cell nuclei remain visible. The volume of the oocyte increases slightly and a further synthesis of yolk granules occurs.

RESULTS

I. *The fixed —SH Groups*

(a) *Oocyte development.* Fixed —SH groups can be demonstrated throughout the ovarian history in both nucleus and cytoplasm of the oocyte, nurse cells, and follicular cells. In the nucleus they are mainly concentrated in the chromatin substances and in the nucleolus. The nuclear sap is negative. In the cytoplasm certain discrete granules always stain more intensely.

In the oocyte the intensity of the —SH reaction increases after stage 3, especially in the cortical ooplasm which stains deep blue as compared with the greenish-blue of the central ooplasm. As maturation proceeds, the distribution of fixed —SH groups becomes more even, but a uniform and higher concentration is still evident in the egg cortex.

In the nurse cells fixed —SH groups are very abundant in the cytoplasm in stages 1–5. Particularly reactive are those areas near the nuclear and vacuolar membranes. Parallel with the decrease of basiphily, the —SH reaction of the nurse-cell cytoplasm weakens in stage 6, whereas the pycnotic nuclei remain strongly positive.

Like the nurse cells, the follicular cells are very rich in fixed —SH groups. In certain stages (3 and 4) an intracellular gradient is evident. The deep-blue granules are accumulated in the proximal half of the cell and concentrated especially near the cell membrane which seems to be connected with the egg cortex by bluish protoplasmic threads. The follicular cells still give a strong —SH reaction in stage 7.

Control sections gave no reaction in most cases. Occasionally the structures containing a high concentration of fixed —SH groups, such as the egg cortex and chromatin materials in nuclei, remained faintly positive after mercuric chloride treatment.

The adult testes are equally rich in fixed —SH groups. Sperm heads are extremely reactive.

(b) *Embryonic development.* A cortical concentration of fixed —SH groups is clearly visible in the early cleavage and blastoderm stages which agrees with the distribution already found in mature oocytes. Mitosis is always synchronous in these stages. If the nuclei of an early embryo happened to be in the course of mitosis at the time of fixation, the cytoplasmic —SH reaction was far more intense than that of a corresponding embryo whose nuclei were not in division. This fact is in accordance with the general view concerning the relation between fixed —SH groups and mitosis (cf. Needham, 1942). Like the chromatin substances in a non-dividing nucleus, mitotic chromosomes invariably show a vivid blue reaction.

The posterior pole plasm and later the cytoplasm of the pole cells are particularly rich in fixed —SH groups. However, the pole cells become less reactive after their invagination.

As gastrulation and germ layer formation proceed, the surface ectoderm (or presumptive hypodermis) acquires a greater share of fixed —SH groups. This hypodermal concentration, as a consequence of the cortical localization in the unfertilized egg, is traceable in all parts of the surface ectoderm of the embryo. It is still noticeable at the time of the contraction of the germ band, which takes place at about the 9th hour after laying at $25 \pm 0.2^\circ \text{C}$. It should be pointed out that the temporal course of the morphogenetic events as described here is earlier than those given by Poulson (1937), since the whole embryonic life is shortened by about 3–4 hours at $25 \pm 0.2^\circ \text{C}$. as compared with $22\text{--}23^\circ \text{C}$.

Although the mesoderm, endoderm, and the nervous system rudiment are originally derived from the same lot of blastoderm cells which give rise to the future hypodermis, the former group of tissues contain decidedly less fixed —SH groups than the latter. However, no differences in the intensity of —SH reaction have been noticed between the mesoderm, endoderm, and nervous tissue rudiment themselves.

In later embryos (12 hours or older) an increase of —SH reaction has been observed in certain well-formed organs such as the gut epithelia, salivary gland, gonads, and cuticle. With the exception of the cuticle, the increase of fixed —SH groups seems to be correlated with an increase of cytoplasmic ribonucleic acid.

II. Ribonucleic Acid

(a) *Oocyte development.* In the stage 1 follicle, nurse cells contain more ribonucleic acid than either the oocyte or follicular cells. In the next three stages (2–4) an enormous increase of cytoplasmic ribonucleic acid occurs in the nurse cells. They now stain deep red with pyronin. The acid content starts to decrease in stage 6.

The follicular epithelial cells give as intense a staining reaction as do the nurse cells in stages 2–5. They retain most of their ribonucleic acid in stages 6 and 7, a fact which is possibly related to the formation of the chorion and filaments of the egg.

At most developmental stages the oocyte cytoplasm takes less pyronin than the nurse cells or follicular cells. The cytoplasm of younger oocytes (before stage 2) often contains relatively more ribonucleic acid than that of older oocytes (stages 3 and 4). In these latter stages the decrease of the acid content is mainly due to a rapid increase in the volume of the developing oocyte. Nevertheless, cytoplasmic materials at the end of the oocyte adjacent to the nurse cells take pyronin as strongly as does the nurse cell's cytoplasm. This indicates the beginning of the nurse-cell absorption. In stages 5–7 the ribonucleic acid content of the egg shows a definite increase as a result of the direct incorporation of all nurse cells, even though the increase in egg volume is more marked than before. The acid content of mature oocytes decreases very sharply again. This is probably connected with further synthesis of yolk materials before maturation. Pasteels (1948) has recently found that in *Ascaris* oogenesis the decrease of cytoplasmic basiphily is correlated with yolk

formation. Moreover, Brachet (1942) has mentioned that ribonucleic acid decreases during the oogenesis of *Drosophila*. In the light of the present evidence, Brachet's statement is true only when the comparison is made between certain definite developmental stages.

Ribonucleic acid is not demonstrable in the oocyte nucleus at any developmental stage.

Treatment with ribonuclease completely abolishes the cytoplasmic affinity for pyronin. However, the nucleoli of the follicular cells and corresponding structures of the nurse cells were found to retain some stainability after the enzyme action. Pycnotic nuclei of the nurse cells (in stages 6 and 7) show various shades of colour (green, purple, red, &c.) both in the control and enzyme-treated sections. This probably indicates that depolymerization of desoxyribonucleic acid occurs during the nuclear degeneration, since pyronin will stain this acid only in its depolymerized form (Kurnick, 1947).

The testes from freshly emerged or adult males contain a negligible amount of ribonucleic acid in comparison with the ovaries: only the young spermatogonia located at the anterior end of the testis retain a high ribonucleic acid content. The spermatocytes, spermatids, and mature sperm lose their ribonucleic acid in successive degrees until the mature sperm are practically negative to pyronin. This progressive decrease of basiphily during spermatogenesis is particularly clear when prepupal or pupal testes are studied. A similar phenomenon is known in amphibians (Brachet, 1942).

(b) *Embryonic development.* In the newly laid eggs the distribution of ribonucleic acid is diffuse. As soon as the cleavage nuclei begin their migration to the egg periphery, there occurs a simultaneous segregation of ribonucleic acid. Thus, in the incipient or single blastoderm stage, the whole surface blastoderm is so heavily stained by pyronin that it seems as if the ribonucleic acid content is increased. Especially noticeable is the concentration of the acid in the so-called 'innere Blastema' which is later to be partially incorporated with the outer layer of blastoderm cells. No ribonucleic acid is now left in the central yolk except in the thin protoplasmic areas surrounding the yolk nuclei.

Between gastrulation and the contraction of the germ band (3-9 hours embryos) most principal organ rudiments have been laid down. During this period no conspicuous differences in ribonucleic acid content between different germ layers and their derivatives have been observed. Within the ectoderm itself, however, a differential distribution exists—the half of the cell facing the yolk always contains more ribonucleic acid than the other half. Since this condition was found in the ventral, lateral, and dorsal ectoderm as well as in the invaginating stomodaeum and proctodaeum, it is possibly a mere consequence of the concentration of ribonucleic acid in the 'innere Blastema' in the blastoderm stage.

Comparing the degree of basiphily of the embryonic cells of various developmental stages during the first half of embryonic development (0-9 hours), it was found that ribonucleic acid does not show any appreciable

decrease during this period, although no persistent increase of basiphily has been observed as in the case of chick embryogenesis (Brachet, 1947b).

In older embryos (12–15 hours) a differential increase of ribonucleic acid due to differential acquisition of synthetic activity arises. For example, the mid-gut seems to acquire this ability soon after coiling of the intestine has taken place (between the 11th and 12th hour). Other organs whose ribonucleic acid content is comparable to that of the mid-gut are the proventriculus, gastric caeca, salivary glands, and to a lesser extent the hind-gut. The increase of basiphily in the gut, when it begins to differentiate, has also been reported in the early development of sea-urchin (Brachet, 1947b) and of *Limnea* (Raven, 1946) eggs. Organs such as the Malpighian tubes, muscles, fat bodies, and hypodermis contain less ribonucleic acid, though their cytoplasm is still fairly basiphilic. Nerve cells possess the least amount of ribonucleic acid at most stages: the nerve-fibre region is devoid of it.

Between the 15th and 18–19th hours, when the larva hatches, a definite decrease of ribonucleic acid has been observed in most organs. However, the gut epithelia and salivary glands retain their high acid content.

III. Desoxyribonucleic Acid

(a) *Oocyte development.* Using the Feulgen reaction, Painter and Reindorp (1939) have made a detailed study of nuclear phenomena in the nurse cells of *Drosophila* ovaries. Analysis of the very early follicles in our preparations has confirmed their finding of the general phenomenon of endomitotic growth in the nurse cells. A few points which are relevant to the development of the oocyte as a whole will be mentioned here. In the first place, there seems to be some relation between the physiological activity of the nurse cells and their degree of polyteny. Morphological and cytochemical evidence each indicates that the nurse cells are physiologically most active in the stage 4 follicle. The nuclear diameter of the largest nurse cell at this stage has already reached 40μ which is at least 512-ploid according to Painter and Reindorp. Secondly, the reactivity of the nurse-cell nucleus as a whole towards Schiff's reagent and the number of the Feulgen-positive granules around the nuclear membrane decrease sharply in stage 5. This is then followed by nuclear pycnosis and reassumption of Feulgen positivity. Lastly it may be stated that there is no demonstrable change in the Feulgen reaction of nuclei of either the follicular cells or the oocyte throughout the ovarian history.

(b) *Embryonic development.* A rough inspection of the intensity of Feulgen reaction in developing embryos of various stages indicated that there can be no large-scale synthesis of desoxyribonucleic acid after the stage of the germ-band contraction. This contention was confirmed by an actual study of mitotic frequency. It was found that mitosis occurs most frequently between the 5th and 8th hours after laying at $25\pm 0.2^\circ\text{C}$. After the 9th hour cell division can be seen in the brain, mesenchyme cells (?), and occasionally in the ventral ganglion; but not in other tissues. This result is in entire agreement with the findings of Poulson (1945).

The Feulgen reaction of advanced embryos (10–18 hours old) does not differ very much from that of the embryos at the time of the germ-band contraction (9 hours). The only difference is found in the nervous system which, by virtue of the small size of the cell nuclei and the continuous reduction in the volume of the organ as a whole, stands out more sharply in the later than in the early embryos.

Since the growth of the larval tissues in *Drosophila* takes place almost exclusively by the process of endomitosis (Cooper, 1938), it would be interesting to know whether endomitotic growth starts during embryonic development. The nuclear size of the cells of mid-gut and salivary glands has thus been compared between the stages immediately after the contraction of the germ band and just before hatching. Although the nuclear size in the latter stage is definitely larger than that in the former, the total increase in nuclear volume amounts to less than 50 per cent. (from about $28\mu^3$ to $40\mu^3$ for the salivary gland cell nuclei). If the relation between the nuclear division cycle and doubling of nuclear volume holds true, this would suggest that endomitosis does not occur during embryonic life.

DISCUSSION

The importance of fixed —SH groups and ribonucleic acid in the determination of the embryo axis in vertebrate development has been particularly emphasized by Brachet (1947a). From the present study there is no reason to suppose that such substances also play a prominent role in *Drosophila* embryogenesis. Some concentration of fixed —SH groups and a differential distribution of ribonucleic acid have been found in the ectoderm of early *Drosophila* embryos. However, owing to the general occurrence of these properties, it is difficult to attach any morphogenetic implications to them. Raven (1946) found a similar situation in *Limnaea*.

Cytochemical evidence indicates that there is no detectable decrease of basiphily during the first half of embryonic development. This maintenance of a relatively strong basiphily must be due to some synthesis of ribonucleic acid, for otherwise one would expect a gradual decrease of basiphily parallel to the increase in cell number and the scale of organization of the embryo, as in the case of the sea-urchin (Brachet, 1947a, b). Nevertheless, since a small change of ribonucleic acid content (giving a small decrease in staining intensity) might have escaped notice, the possibility of the conversion of some ribonucleic acid into desoxyribonucleic acid is not excluded by the present observation. In fact, Brachet (1947c) has recently cited some unpublished data that such conversion does occur in some insects. The apparent resemblance between the nucleic acid metabolism of the *Drosophila* embryo and that of the chick embryo must, therefore, await direct confirmation or possibly even disproof.

As histo-differentiation goes on, there is generally a gradual decrease of ribonucleic acid. This is particularly clear in *Drosophila* embryos older than 15 hours (at $25 \pm 0.2^\circ \text{C}$). In certain organs, such as the salivary glands and gut

epithelia, however, an accumulation of ribonucleic acid occurs in the course of differentiation. This is possibly related in some way to the normal physiological function of these organs and may well be the result of histo-differentiation.

The very fact that mitosis is of rare occurrence after the contraction of the germ band and the lack of evidence for endomitotic growth during the latter half of *Drosophila* embryogenesis leads me to suggest that the stage of 'germ-band contraction' marks the transition from a growth phase of embryonic development into a differentiation phase. The term differentiation used here refers to visible histo-differentiation rather than to invisible chemo-differentiation. The latter takes place well before 'germ-band contraction', as indicated by Geigy's (1931a, b) work.

SUMMARY

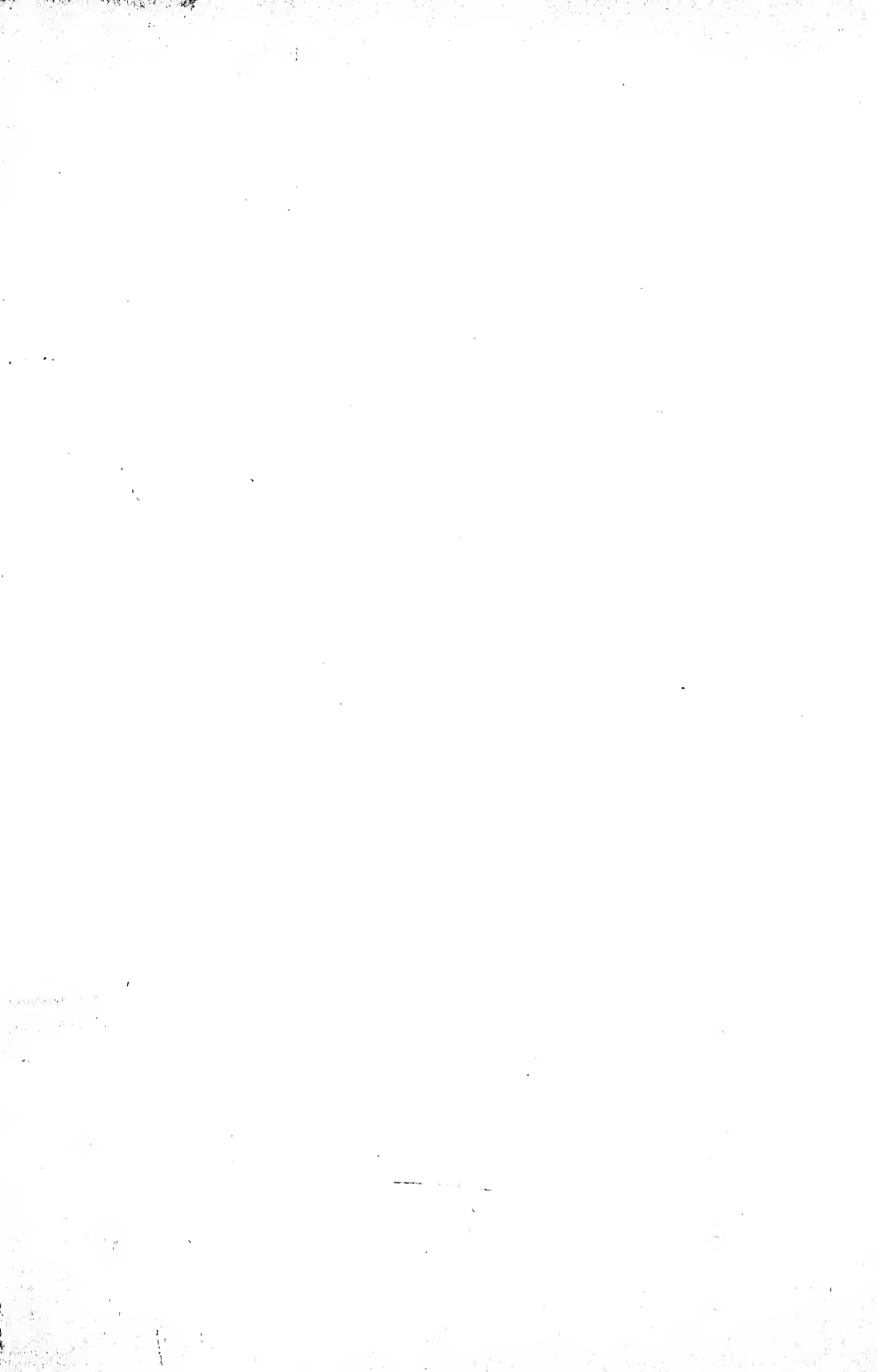
In the egg of *Drosophila* the distribution of sulphhydryl and ribonucleic acid compounds has no apparent connexion with the dorso-ventral organization, contrary to the situation found in the amphibian egg.

Histochemical evidence suggests that the nucleic acid metabolism of the *Drosophila* egg may be similar to that of the chick embryo.

During embryogenesis the contraction of the germ band can be considered as an important morphogenetic stage which marks the beginning of the histo-differentiation of all larval structures.

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A Critique of the Plasmal Reaction, with Remarks on Recently Proposed Techniques

BY

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INTRODUCTION

THERE has been much disagreement between histochemists and biochemists over the chemical basis of the plasmal reaction and the techniques to be employed in demonstrating it. The purpose of this paper is to provide a critique of the methods that have been proposed.

INVESTIGATIONS BY THE FEULGEN SCHOOL

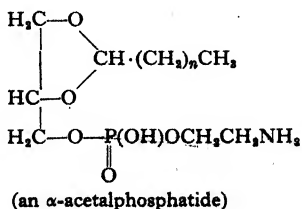
The occurrence in cytoplasm of substances giving a positive result with Schiff's reagent ('fuchsine-sulphurous acid') was first announced by Feulgen and Rossenbeck (1924, p. 230) in a footnote to a paper on the nucleal reaction. In the same year Feulgen and Voit showed that the substances responsible, which were adsorbed on to certain elastic-tissue elements so strongly as to resist paraffin embedding, were aldehydes, as the reaction could be abolished rapidly by phenylhydrazine and by sodium bisulphite. Similar substances found in frozen sections of fresh tissue were demonstrated to be lipid by their solubility in alcohol; and Voss, who studied the resistant substances (1927, 1928, 1931*a* and *b*), showed (1927, p. 586) that the longer the tissues were in alcohol, the weaker the reaction became.

Feulgen and Voit tried the effect of mercuric chloride on tissues and discovered that the reaction became intensified, or appeared in tissues previously negative; but the same final result could be obtained by the prolonged action of Schiff's reagent, even after treatment with phenylhydrazine, the phenylhydrazone being decomposed by the acidity of the reagent. In tissues negative without the action of mercuric chloride, a preliminary treatment with alcohol [Quarterly Journal Microscopical Science, Vol. 90, part 4, December 1949.]

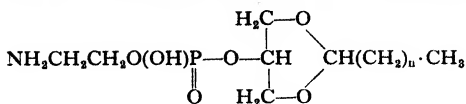
abolished the positive reaction after mercuric chloride. Therefore there was a lipoidal precursor which gave rise to the aldehydes, rapidly under the influence of mercuric chloride, slowly in acid media. They named the aldehydes 'plasmal' to mark the distinction in topography between this and the 'nuclear' reaction; the precursor they named 'plasmalogen'. Extracts of tissues were made, the reaction was obtained *in vitro*, and the thiosemicarbazone silver salt of the aldehydes was obtained in the pure state.

An important series of papers followed, on the occurrence of plasmalogen and plasmal in the body fluids, on their ingestion with food (Voit, 1925; Stepp, Feulgen, and Voit, 1927; Feulgen, Imhäuser, and Westhues, 1928; Imhäuser, 1928), and on their quantitative estimation in body fluids (Feulgen and Imhäuser, 1927; Feulgen and Grünberg, 1939), but these are not specially significant from the point of view of histochemistry. But Imhäuser in 1927 carried out a survey of the occurrence of plasmalogen and plasmal in tissues, and concluded that they were very widespread indeed, in fact that all animal cells contained them, and therefore they must be of fundamental importance in the physiology of the cell. The literature up to 1929 was reviewed by Grevenstuk (1929).

The chemistry of plasmal and plasmalogen was further investigated with great success. Palmital and stearyl were investigated by Feulgen and Behrens (1928), who with Imhäuser showed (1929) that plasmal could be isolated and identified by comparison as palmital with an admixture of some stearyl. But one other aldehyde at least was present which could easily be detected by its strong smell. This aldehyde could not be isolated, and they believed it to be unsaturated. They had discovered that plasmalogen was always found in the phospholipine fraction on extraction, but it was not itself a phospholipine, or formed from one in the course of extraction, since the content varied according to the source of the extract. Egg yolk, for example, has a very high content of phospholipine and only traces of plasmalogen, whereas the phospholipines of muscle or brain might contain 10 per cent. plasmalogen or more. Separation of the plasmalogen from the phospholipines could be accomplished only by saponifying the latter in alkaline solution; in acid solution, of course, plasmal was rapidly produced. Behrens (1930) prepared plasmal by an improved method, and Feulgen and Behrens (1938) obtained palmitic and stearic acids from it by oxidation. The work was surveyed by Feulgen and Bersin (1939) who showed in addition that plasmalogen could be isolated, though with great difficulty, and that it had the structure of an acetalphosphatide (α or β), that is,



or

(a β -acetalphosphatide)

where the fatty acid radicle is palmityl or stearyl (or the unidentified unsaturated acid). It was observed that the base found was always cholamine. They suggested that choline might replace it, but possibly the acetalphosphatides containing choline were decomposed during the alkaline saponification of the other phospholipines, without which, unfortunately, isolation could not be accomplished. They pointed out also that almost certainly the reason why the acetalphosphatides remained unknown for so long, while the other phospholipines were carefully investigated, was that in the analysis of the phospholipines acid media were invariably used at some stage, and these would destroy the acetal linkages, leaving merely the aldehydes and glycerophosphoric esters. Finally Bersin *et al.* (1941) achieved the synthesis of acetalphosphatide. This appears, unfortunately, to be the last paper published by Feulgen's school; if so, it fittingly terminates their excellent researches.

In the investigation of the occurrence of plasmal, Feulgen and Voit (1924) and Imhäuser (1927) used fresh, unfixed sections, keeping some as controls and treating others with 1 per cent. mercuric chloride. They stuck sections on to slides with albumen, let them dry, and 'fixed' them by quick passage through a flame. They laid very great stress on the necessity for a control section untreated with mercuric chloride—a point that was to be almost completely neglected by other workers on plasmal until Gérard (1935) showed that formaldehyde could be used as a fixative provided it was carefully washed out. This was confirmed by Lison (1936*b*) and the practice was followed by Verne (1947*a* and *b*) but not Verne and Verne-Soubiran (1942). Voss (1927-31*b*) and Verne in earlier and some later papers had used a fixative containing mercuric chloride in order to obtain good fixation, thereby depriving themselves of the control section.

INVESTIGATIONS BY VERNE

The results obtained by the histochemists differ somewhat from those obtained by the Feulgen school, and are not wholly self-consistent. The principal worker on the plasmal reaction has been Verne, who has made a very extended survey of many tissues in different animals, both normal and in various induced pathological states, and has come to extremely important conclusions. He showed (1928*a*) that the plasmal reaction is by no means as widespread as Feulgen and Voit, Imhäuser, and others had thought. Many cells and tissues are completely negative, others (e.g. intestinal epithelium, pancreas, muscle) give only a feeble reaction, but the adrenal, myelin, certain parts of the kidney, and certain cells in the lung are intensely positive. The medulla of the adrenal is weakly positive, the z. glomerulosa, outer part of the z. fasciculata, and inner part of the z. reticularis are strongly positive.

The 'spongiocytes' (fat-laden cells) of the *z. fasciculata* are very weak or negative, as is adipose tissue. He noted that there was a relation between the reaction and lipoids, but no complete parallel. The reaction with the myelin sheath was so intense that he proposed it as a method for colouring the sheath (1928*b*, *d*) after fixing in a mercuric chloride or platinum chloride fixative and cutting frozen sections. On further investigation of plasmal (1928*c*), in addition to noting that sodium bisulphite and phenylhydrazine block the reaction, and that, as mentioned above, platinum chloride can also be used to provoke its appearance, he distinguished three types of lipid inclusion: (*a*) positive with sudan III and negative with plasmal, (*b*) positive with both, and (*c*) negative with sudan III but positive with plasmal—the lipid nature of the last being demonstrated by their solubilities. He made the important observation that by the use of oxidizers, such as potassium permanganate, chromic oxide, or hydrogen peroxide, plasmal-positive bodies could be changed to negative, and plasmal-negative ones to positive, and concluded (p. 268) that such bodies were formed from either neutral fats or phospholipines by oxidation of a suitable hydroxyl group to an aldehyde. He assumes, therefore, that since he has been able to produce positive results by the action of oxidizers on lipoids, those lipoids which already give positive results must already be oxidized in the body and in the same way.

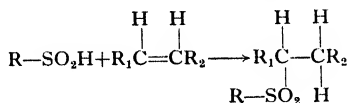
Verne summarized and extended his results in his next paper (1929*a*) in which he makes the interesting remark (p. 248) that fixation should be for at least 6 hours. He points out that, as Feulgen and his collaborators had stressed, a positive result is indicated by a red colour which is much more bluish than that given by Schiff's reagent which has been allowed to become recoloured through the loss of sulphur dioxide. He notes that sebaceous glands are negative, that the interstitial cells of the testis are positive, and that the adrenal is the only other endocrine gland studied which was positive. His results are based on studies of man, dog, cat, cow, calf, sheep, chick, common frog, tench, and eel. In experiments with oxidizing agents he found that adipose tissue can be made positive with plasmal, and that in general, as a lipid body becomes positive through oxidation the intensity of colouring which it will assume on treatment with sudan III progressively decreases and becomes negative. Sections of adrenal left in water with a trace of mercuric chloride showed a positive result in the *z. fasciculata*; the same effect was produced in a quarter of an hour by 0.5 per cent. permanganate, or after several hours with 1 per cent. chromium trioxide. He mentions certain renal tubes and pulmonary cells and the adrenal medulla as negative to sudan III and positive to plasmal, and remarks that the pulmonary cells will withstand paraffin embedding. Researches on pure substances showed that pure glycerides and fatty acids (he does not say which), and cholesterol are always negative. Oleic acid and triolein are negative when pure, but become positive after exposure to the air. Unsaturation appears to be necessary, except that saturated aldehydes will give the reaction; unsaturated aldehydes are not present since they cannot be regenerated from the sodium bisulphite com-

pound by dilute acid, whereas the reaction can be made to reappear in tissues after treatment with sodium bisulphite. Consequently he revises his previous conclusion that the aldehydes concerned are formed from alcohols, and now considers that they are formed by oxidation at double bonds, which would explain why it is only the saturated aldehydes that are in question. As aldehydes are notoriously reactive, they are probably in some sort of labile combination in the tissues (those, that is, that have already been produced *in vivo*). The combination is broken by the action of the mercuric chloride or platinum chloride in the fixative. Free aldehydes, he states, have never been found in the tissues. He tried the action of mercuric chloride on various lipoids *in vitro* but could obtain no result.

Verne has not departed from these conclusions, but has strengthened his evidence for them in a long series of papers: on the adrenals (1929*b*), on the action of carotenoids in protecting lipoids from oxidation (1936*a, c*), on the acceleration of oxidation by glutathione and colchicine (Verne and Verne-Soubiran, 1939), and especially on the lipoids of the kidney (1937*a, c*, 1940).

INVESTIGATIONS BY LISON AND GÉRARD

However, certain objections to the specificity of the plasmal reaction had been raised. Lison (1932), after a very careful study of Schiff's reagent, showed that it was by no means specific for aldehydes, although aldehydes were among the most familiar compounds that would give a positive result. He reviewed the work of Wieland and Scheuing (1920) who had finally established the nature of the reaction involved with aldehydes, and concluded that the reaction took place with double bonds in other substances besides aldehydes. It is an addition reaction of the type:



He disagrees with the Feulgen school (Feulgen, Imhäuser, and Behrens, 1929), who claimed that the reaction is a pseudo-reaction with acetone; and he states, in contradiction of Verne (1929*a*), that oleic acid does give a positive reaction, though only after 15-45 minutes. He makes two very important comments: firstly, substances which are not aldehydes and yet give a true reaction will also react with sodium bisulphite, phenylhydrazine, and other reagents supposed to identify aldehydes, and secondly, that the reaction of any such substance with one of these was no guarantee that it would react with others. Consequently, if an unidentified substance in tissues reacts both with Schiff's reagent and with phenylhydrazine, it is not characterized thereby as an aldehyde, nor does it follow that it must react with, say, semicarbazide. This paper is of the highest importance in the interpretation of results obtained with Schiff's reagent.

Lison also remarked that Schiff's reagent could be recolored by oxidant

enzymes if oxygen were present. He was not sure of the interpretation of the colours produced, and thought that the process was probably a pseudo-reaction. Gérard (1935) studied the distribution of plasmal and of 'oxidases' (apparently peroxidases and phenolases according to Lison, 1936*b*, Chapter VI). He used dimedone (dimethyl dihydroresorcinol) to block all aldehyde radicles but found that it did not block the plasmal reaction. Further, there was a complete parallel between the plasmal reaction and the reactions for 'oxidases' except in myelin, and he found that Schiff's reagent was readily recolored by iodates, periodates, and other oxidizers. As phenylhydrazine acetate does block plasmal, and as the Nadi reaction was positive even after treatment with cyanide (which would seem to rule out an oxidant enzyme) he inferred that there was an oxidizing agent in the lipoids; this was confirmed by the observations that oxidized oleic acid would liberate iodine from slightly acidified potassium iodide (as would sections) and would recolorize some of the reagents for 'oxidases'. Myelin explicitly excepted, his conclusion is (p. 278) that the plasmal reaction when given by inclusions shows a secondarily acquired oxidizing system.

Lison (1936*a*) also studied the Nadi reaction, and noticed that some fats give a feeble positive result with it, which he considered to be due to a direct reaction as it was not inhibited by cyanide, and was improved on boiling. He confirmed Gérard's findings, and added that the substance concerned would give a positive result with benzidine and a peroxidase, and so must be a peroxide. It is formed by atmospheric oxidation at a double bond.

Verne had shown (1937*a*) that in the kidney of the dog there were lipid bodies that were positive with plasmal, but negative with Nadi, and that tissues which gave a coloration with Schiff's reagent when 'fresh' and after formaldehyde-fixation and treatment with mercuric chloride, did not do so if mercuric chloride were not used. Formaldehyde, in fact, would block the recoloration. He answered the objections raised by Gérard and by Lison, in two papers (1937*b*, 1940) repeating the results just mentioned, and adding that osmium tetroxide, and iridium, platinum, and gold chlorides could be used after formaldehyde-fixation as well as mercuric chloride. (Oster and Schlossman (1942) appear to have discovered the use of gold chloride independently.) His conclusion was (1937*b*, p. 276) that the Nadi reaction demonstrated the progress of autoxidation of the lipoids, whereas the plasmal reaction showed the appearance of products resulting from this autoxidation, which took place at double bonds. Consequently, the lipoids showing only the plasmal reaction ('Feulgen-Verne reaction') must be saturated, all the double bonds being destroyed. Finally he remarks that aldehydes have been detected in rancid oils.

In his second paper (1940) he showed that the plasmal reaction was inhibited by carbon monoxide and cyanide but not by ethyl-urethane. Sections of adrenal and kidney in contact with ethyl-urethane for 24 hours gave a positive reaction as if they had been in water. The reaction could be regenerated after cyanide by mercuric chloride. He thought, therefore, that the oxidation

must be mediated by a polyphenol-oxidase. As Lison had shown that the Nadi reaction was unaffected by cyanide, this strengthened Verne's views on the separate natures of the two reactions. Further, the plasmal reaction was blocked by dimedone and could not be regenerated by mercuric chloride. This result was in complete contradiction of Gérard, and Verne considered it as disposing of Gérard's objections. He also claimed that he had obtained on at least one occasion a reduction of ammoniacal silver nitrate by lipid inclusions, which he took to demonstrate with certainty the presence of aldehyde groups.

METHODS AND INTERPRETATIONS

The results of the histochemists are indeed far from presenting the same simplicity, clarity, and coherence as those of the biochemists; nor do they lead to the same conclusions. Also, the technique employed has been varied considerably. There appear to be several distinct reactions, which may or may not have the same basis. The reader may find it convenient at this stage to turn to the table on p. 419. The techniques have been summarized by Cain (1949).

The plasmal technique (that of Feulgen and his colleagues) appears unexceptionable, but unfortunately its users attached sections to slides with albumen by allowing them to dry, and then 'fixing' by passing through a flame. In view of Verne's conclusions on the importance of oxidation at double bonds, it might be objected that the very wide distribution of plasmal as found by the Feulgen school is due at least in part to their method of attaching their sections, provided that the oxidation, once started, is catalysed by mercuric chloride, and by acidity. Also there is the possibility of interference by oxidizers of various sorts as shown by Gérard, and by Lison (1936a), provided they are not destroyed by the heating.

The Feulgen-Verne technique has the great disadvantage that there is no control section. Consequently it cannot distinguish between those aldehydes or oxidizers (if any) already present in the tissues and those revealed after the action of mercuric chloride. Yet plasmal is described by its discoverer as being liberated under the influence of mercuric chloride from acetal-phosphatide. It follows that the F-V technique cannot be called a 'plasmal' technique.

The method of Gérard would appear to be the best were it not for Verne's very disturbing observation that formaldehyde can block a reaction, present in 'fresh' tissue, which can be revealed again by mercuric chloride. It appears that when discussing this (1937a, p. 4) he is referring to a recoloration of Schiff's reagent by fresh tissue without the intervention of mercuric chloride. The effect would be to produce a pseudo-plasmal reaction, because if the tissue were really positive when fresh and there was no intensification of the reaction after treating it with mercuric chloride, then the presence of plasmalogen in Feulgen's sense could not be demonstrated for certain; yet it would appear to be present after fixation. However, the 'plasmal reaction'

described by Liang (1947) as being produced by 'fresh' myelin occurs only after several hours' soaking in Schiff's reagent.

The interpretations placed on the different techniques vary correspondingly. The difficulty in evaluating them lies in the fact that lipid droplets in tissues are never pure and may give rise to a variety of reactions, few of which are really connected. The best evidence is obtained when a reaction is prevented by preliminary treatment with a certain reagent, but there is no guarantee that the reagent is not reacting with other radicles or components at the same time.

Feulgen's interpretation is simple. Acetalphosphatides are decomposed, extremely rapidly with mercuric chloride, to give palmital, stearal, and other higher aldehydes, which give a positive result. Acetaldehyde, studied in urine by Stepp and Feulgen (1921, 1922), occurs in too small quantities to give positive results. It should be noted here that many authors speak of a positive result as the recolouring of Schiff's reagent. This is perfectly true in one sense—the reagent is bleached as it is prepared and now is coloured again—but very misleading in another—the colour is not necessarily or usually the same as that of the reagent recolored on evaporation of sulphur dioxide, that is, of basic fuchsin. However, too great a reliance on the difference in shade is inadvisable. Lison (1932) showed that very various shades were given in quite genuine reactions; certain authors insist that a positive result is always much bluer than a recolouring produced merely by evaporation; but this is not always true, and in addition, the colour can vary according to the mode of viewing. A section which appears magenta when looked at under the microscope by transmitted light, may appear deep violet when lying on the bench.

Feulgen and his school were able to produce the plasmal reaction with mercuric chloride *in vitro*; and they remark frequently (e.g. Feulgen and Bersin, 1939) that the speed of reaction with mercuric chloride is very high—*fast augenblicklich*. Yet nearly always the reactions described by the histochemists require many minutes to develop, even in the presence of mercuric chloride. Feulgen and Bersin (1939) note that the speed depends to a great extent on the degree of dispersion of plasmalogen; but one would expect to find it in a state of very fine dispersion in the tissues, especially since phospholipins are extremely good dispersing agents. The comparative slowness of the reaction in tissues is very suspicious, particularly in combination with Verne's remark that it is necessary to fix for at least six hours; and the possibility of atmospheric oxidation increases with the time spent in manipulating the tissue. A reaction *fast augenblicklich* has not been described by anyone but Feulgen and his collaborators. One wonders whether a genuine and unequivocal plasmal reaction has ever been seen in fixed tissues.

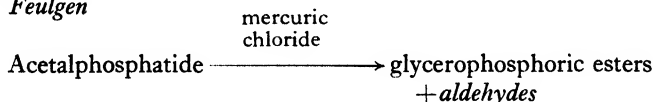
Verne's great contribution has been to show that a positive reaction with Schiff can be provoked by means of oxidizing agents, and prevented by their continued action. Oleic acid, if exposed to the air, will become positive, as will triolein. And Gérard and Lison have shown that it will then

recolorize other leucobases, and that similar substances in tissues definitely appear to form peroxides. Verne maintains, however, that aldehydes are in question. All three agree that the reacting radicle—whichever it is—arises from the double bonds of unsaturated substances. One point requires explanation here. Verne, basing his statements upon the researches of Kaufmann and Lehmann (1926*a, b*), remarks (1937*a, b*) that osmium tetroxide and sudan III colour only unsaturated lipoids. This is only partly true. Osmium tetroxide will not colour directly many compounds (such as cholesterol and lecithin) which contain double bonds, although it may do so after subsequent treatment with alcohol. Sudan III and the other sudan lipid-colorants will (as remarked above) colour all lipoids, saturated or unsaturated, provided only that they are neither combined with proteins in such a way as to mask their lipid nature, nor are they solid—a point which is of great importance here.

It can be shown very easily that sudan black will dissolve in tristearin (for example) *if the tristearin is liquid*. But as the lipoids that are liquids or greases at room temperature, and occur at all commonly in tissues, are invariably unsaturated, Verne is right in practice although theoretically wrong. The reason why he observed lipoids becoming sudan-negative on oxidation would appear to be that the melting-point is being raised by an increase in their degree of saturation.

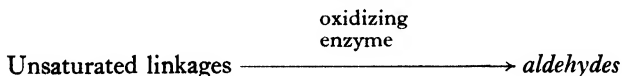
The various theories may be summarized as follows, the positively reacting substance being italicized:

A. Feulgen

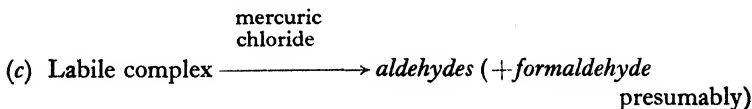
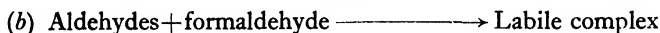
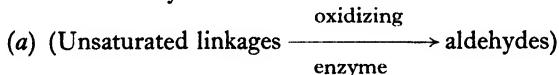


B. Verne

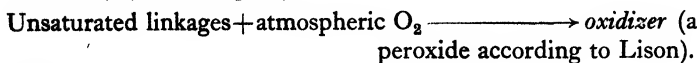
(i) With mercuric chloride fixation



(ii) With formaldehyde fixation



C. Gérard (myelin excepted)



Feulgen's theory has the support of his very impressive biochemical researches on plasmal and plasmalogen, culminating in their preparation in the pure state, and synthesis. From the emphasis he places on the necessity for a control section it would appear that he obtained positive results with fresh tissues, but did not regard them as necessarily showing plasmal; in this he was right, because what is specific in his technique is the *rapid appearance* of positive substances after the action of mercuric chloride (or of acid solutions for a much longer period). Since a mere recoloration of Schiff's reagent is not specific for aldehydes, nothing can be said about a positive result in fresh, untreated tissue.

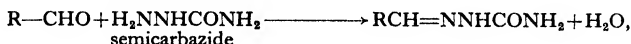
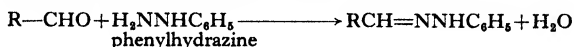
Verne suggested (1937*b*, p. 275) that in some cases in which the reaction is negative with fresh tissues the fixative might break down a lipoprotein complex. Where the fresh tissue is positive, he considered that aldehydes are concerned, and that these had been formed already in the tissues from unsaturated linkages, by the same method as he had used to produce a positive result after fixation, that is, by oxidation. The production of positive results with the Nadi reagent and leucobases he interpreted at first (1937*b*) as merely showing that auto-oxidation was proceeding, but later (1940) as showing the presence of an enzyme (polyphenoloxidase) catalysing the reaction.

According to Lison (1936*b*, Chapter XVI) the subject of enzymes is one of the most confused in histochemistry. What is certain is that those enzymes that can be demonstrated cytologically catalyse the oxidation of polyphenols, polyamines, and other substances that are not themselves found in tissues; and there is no evidence whatever that such enzymes will catalyse the oxidation of other substrates. They cannot catalyse normal physiological oxidations. He adds that on the other hand one cannot say that these enzymes have nothing whatever to do with cellular respiration, because 'dans ce domaine, l'obscurité est encore totale'. Verne's evidence for the action of an enzyme (namely that the plasmal reaction is blocked by carbon monoxide and by cyanide but not by ethyl-urethane) is insufficient. It is necessary to show that these substances do not by themselves prevent the oxidation of double bonds. It is at least likely that, being themselves unsaturated, they can form addition compounds at these linkages, so protecting them from atmospheric oxygen, *in vitro*.

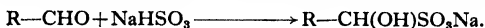
It seems a little unjustifiable, also, to assume that a positive result in fresh tissues is necessarily due to the formation of aldehydes by the oxidation of double bonds. So very little is known of the intermediate metabolism of fatty radicles; what is known chiefly concerns the β - or ω -oxidation of saturated acids. Smedley-MacLean (1943) considered that there is no evidence that the unsaturated acids represent stages in oxidation of fatty acids, although her view is contested by some (see the review in Hilditch, 1947). It is never possible for the histochemist to anticipate the biochemist in identifying unknown radicles in tissues unless he can prove his reagents to be specific.

The specificity of the plasmal reaction depends on the catalytic action of mercuric chloride, and the specificity of the reagents used to produce or block

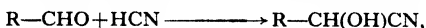
it, namely, phenylhydrazine and derivatives, semicarbazide and derivatives, sodium bisulphite, carbon monoxide, cyanide, dimedone, ammoniacal silver nitrate, and of course Schiff's reagent. The reaction of phenylhydrazine, semicarbazide, and their derivatives is a condensation, an intermediate addition product which is unstable being usually postulated,



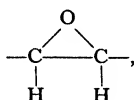
whereas the reaction with sodium bisulphite is a simple addition,



Hydrogen cyanide, always present in solutions of its salts, will react similarly,

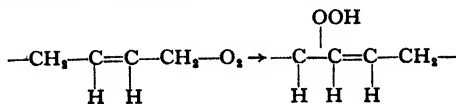


so that the blocking of the plasmal reaction by cyanide need not require the inactivation of an enzyme. These additions are reminiscent of the additions of Schiff's reagent to double bonds, and in fact sodium bisulphite will react with some $>\text{C}=\text{C}<$ linkages (see e.g. Hickinbottom, 1948). It might be thought that the phenylhydrazine or semicarbazide condensations, which apparently require a $\text{C}=\text{O}$ linkage might be specific. Unfortunately, these compounds are also strong reducing agents and soluble to some extent in lipoids. Their presence might well prevent the continuation of oxidations in the same way as do carotenoids. The ammoniacal silver nitrate reagents react, of course, with many reducing agents, of which aldehydes are by far the most likely to occur in lipid inclusions. Unfortunately ethylene oxide groups (Markley, 1947, p. 461) will also reduce them, and the occurrence of such groups has been claimed (e.g. by Szent-Györgyi, 1924) in the oxidation of certain acids, particularly under the influence of $-\text{SH}$ groups, which are the active groups in glutathione. It is not claimed that ethylene oxide groups, which have the constitution:



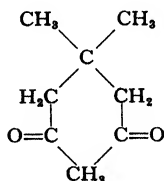
are commonly formed, but their presence must be taken into account. Research on the modes of atmospheric oxidation of unsaturated fatty acids has been carried out principally on oleic, linoleic, and linolenic acids. The very extensive and controversial literature is summarized and discussed by Hilditch (1947) and Markley (1947). Arachidonic acid has been found to occur widely in small quantities, particularly as one of the fatty acid radicles in phospholipines. Ault and Brown (1934) found that it formed over 20 per cent. of the acids in phospholipines from ox adrenal. Unfortunately no studies of its autoxidation appear to be available; as the most highly unsaturated acid present in many animal tissues, it should be of considerable importance in the plasmal reaction if Verne's theories are correct. In general, autoxidation

of oleic and linoleic acids at room temperature appears to begin by the formation of a hydroperoxide group on a carbon atom next to the double link.



This is itself an oxidizer. Further stages involve the opening of the double bond to give keto and hydroxy groups, and finally the chain breaks, giving aldehydes and diacids, the aldehydes then oxidizing to acids as well. Many of the intermediate compounds are unstable and difficult to isolate, and their properties are but little known. The use of heavy metals and their salts in hastening oxidation, probably at first by destroying anti-oxidants (most likely polyphenols), is well known in industry.

If then the unsaturated acids in tissues oxidize readily, it is likely that the hydroperoxide, keto- and hydroxy-radicals, and possibly ethylene oxide groups may all coexist with double bonds. The hydroperoxides could be expected to recolour Schiff's reagent by oxidation and reduce ammoniacal silver nitrate, and to be destroyed by the reducers phenylhydrazine and semicarbazide, which could also prevent further oxidation. It is quite possible that sodium bisulphite might act in the same way, and perhaps add on to double linkages in highly unsaturated chains, as might carbon monoxide and cyanide. Dime-done, which has been used with contradictory results by Gérard and by Verne (1940) is 5:5 dimethyldihydroresorcinol (1:1 dimethylcyclohexandione 3:5) and has the constitution:



According to Heilbron and Bunbury (1934) it is used for characterizing carbonyl compounds, in which case it should react with aldehydes and ketones, the latter being present in partly oxidized unsaturated acids. It also appears to be a reducing agent.

RECENTLY PROPOSED TECHNIQUES

If the oxidation could be catalysed by mercuric chloride, then all the reactions described by cytologists could be accounted for without the necessity for postulating enzymes, or the formation of aldehydes, or the presence of acetal linkages, unless the speed of the plasmal reaction could be shown to be too great to be explained by oxidation. In that case we have two possibilities: either there is a catalysing enzyme also present in tissues, or else the reaction is a true plasmal reaction in Feulgen's original sense.

This question has been investigated recently (Cain, 1949), with the result that a sharp distinction has been drawn between the true Feulgen plasmal reaction and the Feulgen-Verne reaction. The former is due, as Feulgen showed, to the release of higher aliphatic aldehydes (plasmal), presumably from acetalphosphatides, under the catalytic influence of mercuric chloride. Lengthy fixation *reduces* the reaction progressively, and finally abolishes it entirely. A control, uninfluenced by mercuric chloride, is essential. The Feulgen-Verne reaction *increases* in intensity with the length of time in fixative (or other fluids) provided that air has free access; it is not appreciably catalysed, or only slightly, with mercuric chloride; and it appears to be due to oxidation products, perhaps hydroperoxides, formed at points of unsaturation in fatty acid radicles by the action of atmospheric oxygen (or in sterols according to Shear and Kramer, 1926). The evidence brought forward by Verne that aldehydes are responsible rests on the assumptions that all the positive reactions used to prove this point are caused by a single substance in the tissues, and that the reactions used are specific for aldehydes, both of which assumptions are unjustified.

The investigations of Hayes (1947, 1949) support the conclusions reached by Cain, and this author especially emphasizes the necessity for a control section and for rapid handling of the tissues. He finds that formaldehyde-fixation 'rapidly diminishes and finally destroys' the true plasmal reaction and progressively develops a secondary reaction with Schiff's reagent that is not affected by mercuric chloride, and shows a quite different distribution from the true plasmal reaction in some tissues. He concludes that it 'probably demonstrates carbonyl-lipids other than acetals'. This is not quite in agreement with Cain's results, which suggest that other groups, equally produced by autoxidation, may be concerned, and that there is no direct evidence at all that carbonyl groups are the main cause, but the disagreement is slight. The results of these two workers, obtained completely independently, are in substantial agreement.

Hayes prescribes a technique involving the use of frozen sections, which are very inconvenient with some tissues. In Cain's technique (1949, p. 79) this difficulty is overcome by the use of very small pieces of tissue which are exposed while fresh to the action of Schiff's reagent, both with and without mercuric chloride, and then plunged into formaldehyde. The resulting formaldehyde-Schiff compound can be washed out, leaving the plasmal-Schiff compounds in place. Fixation by this method is not good, but it does allow of precise localization of the plasmal reaction within tissues. Penetration by Schiff's reagent is poor, and only very small pieces of tissue can be used.

The investigations of Danielli (1949) do not show results in accordance with those just described. The technique proposed by him (p. 68) is open to serious objection in that the fixative used (and recommended, p. 70) contained acetic acid, and that the time of fixation is given as not less than 2 hours and not more than 5 days. The use of acidic fixing fluids must be avoided in view of Feulgen and Bersin's remark (p. 413 above) that acetal linkages are destroyed

in acid media. The effects of long fixation are invariably bad (Hayes, 1947, 1949; Cain, 1949). Further, it does not appear that control sections were used.

Danielli states (p. 70) that the intensity of the aldehyde reaction and the nature of its distribution in liver sections appeared to be independent of the 'physical nature of the fixative', after a considerable variety of fixatives had been tried, and that variation in the times prescribed for the stages of his technique had little effect (p. 72). This suggests, in view of the criticism just made, that it was the pseudoplasmal reaction that was observed, not the true plasmal reaction.

The supplementary tests used by Danielli to support the results of his technique are:

- (i) extraction of sections with fat-solvents (to demonstrate the lipid nature of the aldehyde);
- (ii) use of azobenzene phenylhydrazine sulphonic acid, which is stated to develop a purple colour with aldehydes;
- (iii) use of ammoniacal silver nitrate solution, which is reduced by aldehydes;
- (iv) use of 2 : 4 dinitrophenylhydrazine, which forms a yellow hydrazone with aldehydes;
- (v) treatment with hydroxylamine, before carrying out the Danielli technique for aldehydes. This, by forming an oxime with the aldehydes, prevents the formation of the aldehyde-Schiff compound.

The results of these tests are considered to prove conclusively that the colour obtained with reduced fuchsin in liver sections is due to the presence in the sections of lipoidal aldehydes. But as it has been shown (Cain, 1949) that the pseudoplasmal reaction is easily prevented by the use of reducing agents which protect unsaturated fats from atmospheric oxidation, and the reagents used in tests (ii), (iv), and (v) are reducing agents, these tests cannot be used to discriminate between a plasmal and a pseudoplasmal reaction. A positive result with test (iii) might possibly be due to the presence of other reducing agents in the tissues, and is given by ethylene oxides (Markley, 1947) which may appear in the pseudoplasmal reaction. Test (i) will of course prevent the pseudoplasmal reaction as well as the plasmal. One cannot regard this group of tests as providing support for Danielli's technique.

SUMMARY

1. The acetalphosphatides which are the precursors of the aldehydes ('plasmal') responsible for Feulgen's plasmal reaction are extremely labile compounds which hydrolyse very rapidly in acid media, and are destroyed more or less rapidly during fixation. The liberation of aldehydes from them is catalysed by mercuric chloride.

2. Ordinary unsaturated lipoids, if exposed to air, become capable of producing a colour with Schiff's reagent, which, unless a control section is

used, can be misinterpreted as a positive result for Feulgen's plasmal reaction. Mercuric chloride has little or no influence over the oxidative rancidity of unsaturated lipids.

3. Any technique prescribed for showing the plasmal reaction must avoid acid media and prolonged fixation and handling of the tissues. A control section is a necessity.

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A Simple Method of Staining the Basement Membrane of Glomerular Capillaries

BY

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IN 1943 Thomas described a haematoxylin phosphomolybdic acid lake which stained collagen and reticulin. This observation has been confirmed and extended by the chance observation that on long standing the lake acquires the power of colouring the basement membrane of the glomerular capillaries.

METHOD

Slices of kidney were fixed in a mixture of a saturated solution of mercuric chloride (9 volumes) and commercial formalin (1 volume). After embedding in paraffin, thin sections (about 3μ) were mounted on glass, the wax was removed, and the staining was carried out in aqueous solution, as follows:

1. Lugol's iodine for 1 minute.
2. Excess of 5 per cent. sodium thiosulphate for 1 minute. Wipe the slide round the section.
3. Wash with distilled water.
4. Add the minimum amount of stain (cf. below) necessary to cover the section.
5. Wash with distilled water after 10 minutes.
6. Dehydrate, clear, and mount.

The stain was made up as follows:

<i>Solution A.</i> Haematoxylin	2.5 gm.
Dioxane	49 ml.
Aerated water	1 ml.

<i>Solution B.</i> Phosphomolybdic acid	16.5 gm.
Distilled water	44 ml.
Glycerol (or ethylene glycol)	11 ml.

Filter solution B. Mix equal volumes of solutions A and B and let the mixture stand for at least 10 weeks.

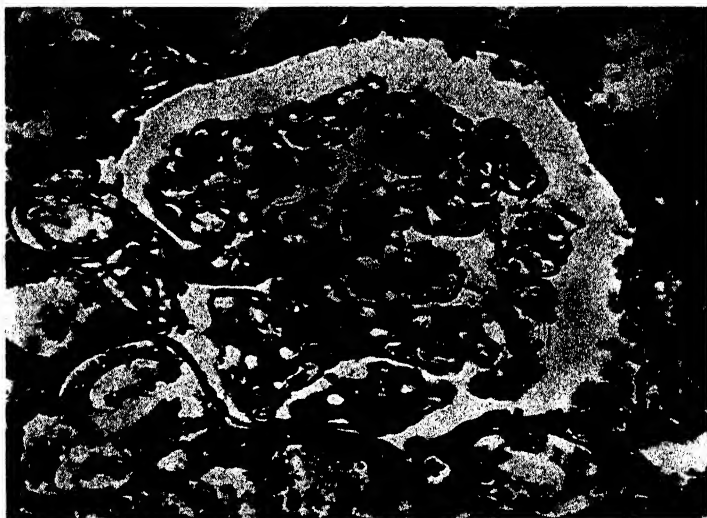
RESULTS

Collagen, reticulin, and the basement membrane of the glomerular capillaries are stained deep violet. Red blood corpuscles are purple. Nuclei are blue, but are lightly stained. The cytoplasm appears as a bluish background.

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The basement membrane of a normal glomerulus appears as a thin, precisely delineated, structure (Text-fig. 1).

The freshly made up stain leaves the basement membrane of the glomerular capillaries uncoloured, though collagen and reticulin are stained violet, as described by Thomas (1943). It is not until the lake has ripened for 3 weeks that weak staining is observed. As ripening proceeds, the intensity of staining increases and becomes adequate in 10 weeks. A sample of the lake now 7



TEXT-FIG. 1. Section of renal glomerulus stained to show the glomerular basement membrane. $\times 340$. Blue-green filter.

months old has retained its power of colouring the basement membrane of the glomerular capillaries.

The nature of the fixative appears to be important because attempts to utilize material fixed in 10 per cent. formol saline were unsuccessful; staining was very diffuse. If the paraffin sections affixed to the glass slides were incubated with saturated aqueous mercuric chloride for 6 days at 37°C . the depth of staining was less than in material fixed in formol mercuric chloride.

As reported by Thomas, only basic counterstains can be used, probably because of the effect of phosphomolybdic acid on cell substances. Acriflavine (1 part) in 1 per cent. acetic acid (5,000 parts) for $\frac{1}{2}$ minute provided a greenish-yellow counterstain and afforded excellent contrast.

COMMENT

This staining method is much simpler than the other well-known stains for the basement membrane of glomerular capillaries, such as the staining methods developed by McManus (1948), Lendrum, Carson, and Penny

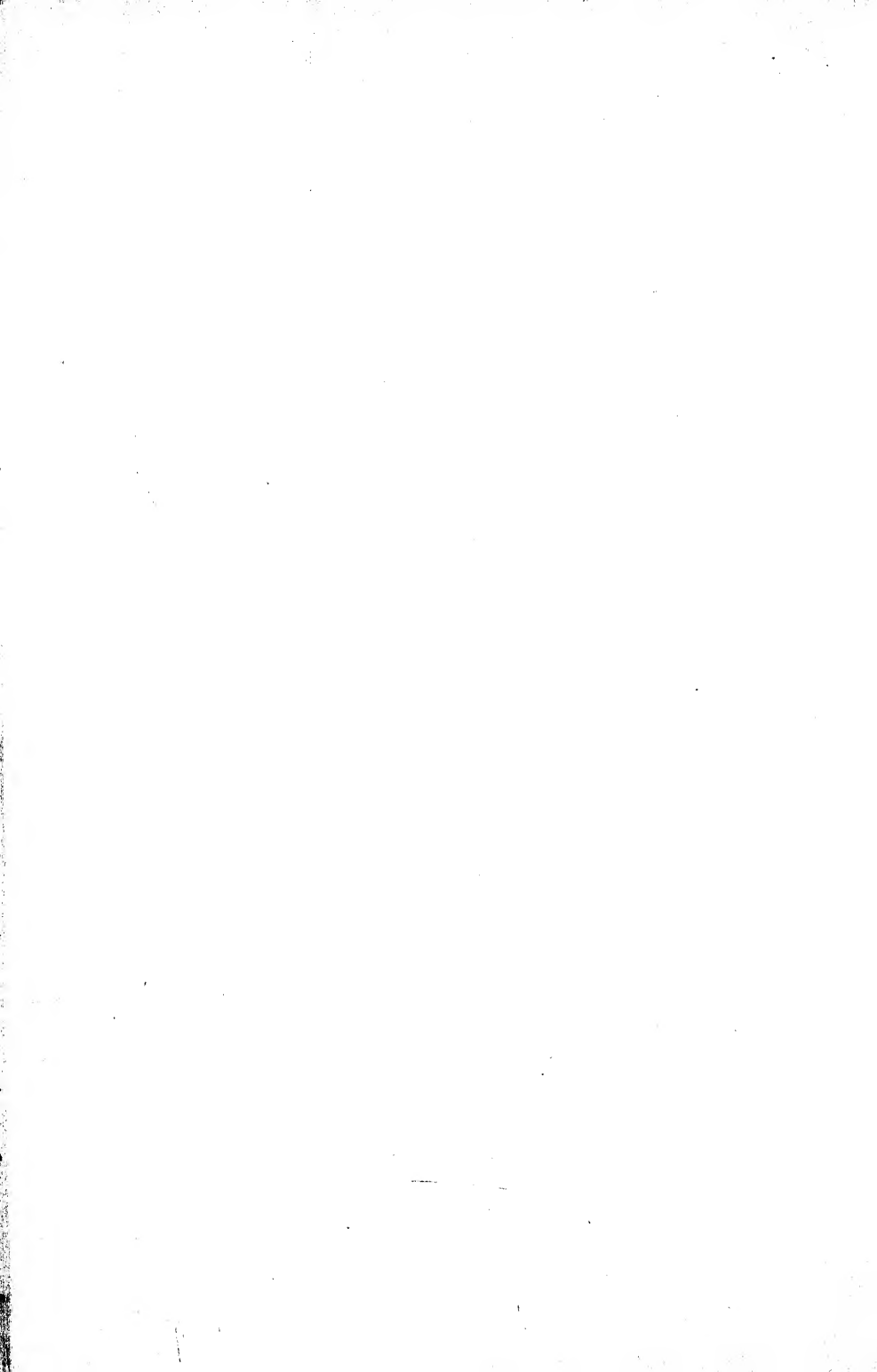
(1945), and Heidenhain (1915). Besides, the clarity of definition of the basement membrane was much greater than when Lendrum's stain was used, or, to judge from the photomicrograph published by McManus (No. 2, p. 649), with his periodic acid-Schiff reagent stain.

SUMMARY

A method of staining the basement membrane of glomerular capillaries is described for tissue fixed in formol mercuric chloride. Ten per cent. formol saline proved to be an unsatisfactory fixative.

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Low Viscosity Nitrocellulose for Embedding Tissues

BY

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LOW viscosity nitrocellulose was introduced in the U.S.A. as a substitute for celloidin by Ruby (1933) and by Davenport and Swank (1934). It is extensively used in that country and has been recommended in text-books on technique by Bensley and Bensley (1938), Conn and Darrow (1947), and Lillie (1948). The advantages claimed are that penetration is quicker and that thinner sections can be obtained.

A similar low viscosity nitrocellulose (L.V.N.) is now obtainable in this country. We have used it by the method described by Bensley and Bensley (1938) and have found it to be most satisfactory. But as the Bensleys also showed it does suffer from certain defects. They have overcome these difficulties (personal communication, 1948) by infiltrating the tissues with L.V.N. and embedding in a tougher sample of L.V.N. with greater viscosity. Such a sample does not seem to be available here.

The defects that we noted, when using L.V.N., were that during handling, staining, and mounting of the sections, the L.V.N. tended to crack and also to break away from the tissues. The addition of a plasticizer, tricresyl phosphate, to the L.V.N. obviates these defects. Lendrum (1941) used the same plasticizer to toughen a similar type of L.V.N. for use in the Peterfi double embedding technique.

TECHNIQUE

Prepare a 20 per cent. solution of L.V.N. with tricresyl phosphate:

Absolute alcohol	210 c.c.
Ether (anaesthetic)	250 c.c.
Tricresyl phosphate	5 c.c.

Mix well and then add 140 gm. of 'Industrial Nitrocellulose damped with 7 : 3 Butyl alcohol. HX. 30/50'. This can be obtained from Imperial Chemical Industries (Paints Division), Wexham Road, Slough, Bucks., or from Hopkin & Williams Ltd., 16 St. Cross Street, Hatton Gardens, London, E.C. 1, or from E. Gurr, 108 Waterford Road, Walham Green, London, S.W. 6. The L.V.N. dissolves quickly and should be ready for use on the following day.

Prepare a 20 per cent. solution of L.V.N. similarly but omitting the tricresyl phosphate.

Prepare a 10 per cent. solution of L.V.N. by diluting the 20 per cent. solution with equal parts of a mixture of ether and absolute alcohol (equal parts).

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Prepare a 5 per cent. solution of L.V.N. by diluting 1 part of the 20 per cent. solution with 3 parts of a mixture of ether and absolute alcohol (equal parts).

PROCEDURE FOR EMBEDDING TISSUES

1. Fix and dehydrate the tissues as usual.
2. Ether and absolute alcohol (equal parts)—1 day.
3. Five per cent. L.V.N.—3 to 5 days.
4. Ten per cent. L.V.N.—1 to 2 days.
5. Twenty per cent. L.V.N.—1 to 5 days.
6. Embed in the 20 per cent. L.V.N.—tricresyl phosphate solution. The paper box should be large enough to leave a margin of at least one quarter of an inch on all four sides of the tissue.
7. Allow to harden slowly in a desiccator. L.V.N. solutions harden more quickly than celloidin solutions. A small folded piece of paper under the lid of the desiccator allows enough ventilation. In 1 to 3 days the block should be adequately hard. At this stage it should be a stiff but easily deformable gel not altered in shape or size by shrinkage; it should be considerably less hard than a celloidin block is usually made. If it is allowed to harden too much or too fast the block starts to shrink and air bubbles may be forced into the block. If the block is too hard the sections will tend to roll.
8. Plunge the block into 75 per cent. alcohol. Change the alcohol at least twice over a period of 1 to 3 days. The block now becomes very hard.
9. Trim the block, removing the hard outer rim of the L.V.N. Use 20 per cent. L.V.N. solution to mount it on the wood or fibre block. Hardening is complete in a few minutes. Dip into 75 per cent. alcohol for a few more minutes.

PROCEDURE FOR CUTTING SECTIONS

Cut the sections 'dry'. If a celloidin microtome is used, the tilt of the knife should be the same as that used for cutting celloidin. But the angle the knife makes with the direction of travel should be between 25° and 45° instead of the usual 75° used for celloidin sectioning. This prevents rolling of the sections. It is possible to cut large blocks, such as half a cat's brain, at 15μ . Small blocks, 5×5 mm., can be cut at 5 to 7μ .

Sections can be cut at least as well on a paraffin microtome without any special modification or attachment. Spencer rotary and the small Cambridge rocking microtomes have proved satisfactory. The large Cambridge flat-cutting microtome seems to be ideally suited for this work, particularly for very thin sections of small blocks. It is comparatively easy to get 3 or 4μ sections even of hard material. The sections can be made to ribbon by coating the upper and lower surface of the block with soft paraffin.

If very thin sections of small blocks are required it may be found advantageous with some but not all tissues to omit the tricresyl phosphate from the 20 per cent. L.V.N. used for embedding. But larger blocks always cut better if the plasticizer is added.

PROCEDURE FOR HANDLING SECTIONS

1. Collect the sections in 75 per cent. alcohol; handle and stain as usual. Dyes tend to stain L.V.N. less than they do celloidin.

2. Mount the sections on to a slide from a bowl of 96 per cent. alcohol. Flatten with toilet paper moistened with the same; press the toilet paper with a glass rod and then remove it. Repeat this several times.

3. Treat similarly several times with equal parts of absolute alcohol and chloroform.

4. Treat similarly several times with the following mixture:

Xylene	2 parts
Toluene	1 part
Creosote	1 part

5. Treat similarly several times with xylene.

6. Mount in balsam.

The following alternative method is somewhat more difficult to use but gives better results with somewhat wrinkled sections of small blocks.

1. After staining, dehydrate the sections.

2. Transfer the sections to 96 per cent. alcohol.

3. Then place them in a mixture of equal parts to absolute alcohol, xylene, and chloroform.

4. Transfer single sections to a deep bowl of beechwood creosote and immediately float them on to a slide. If allowed to stay for more than 2 to 3 seconds in the bowl they will become too soft. Blot the sections on to the slide with toilet paper and smooth with a glass rod. Remove the toilet paper carefully. Immediately cover with another bit of toilet paper, smooth it and then remove it.

5. After removing as much creosote as possible, treat several times with xylene, blotting on as usual.

6. Mount in balsam.

NOTES

1. Low viscosity nitrocellulose is more explosive than celloidin and should be handled with care. When dry it would explode if hit. Exposure to direct sunlight should be avoided.

2. Slides may be coated with L.V.N. instead of celloidin. But a diluted tricresyl phosphate mixture must be used, otherwise the coating does not adhere well to the glass.

SUMMARY

Low viscosity nitrocellulose may be used as a cheap and effective substitute for celloidin. A plasticizer, tricresyl phosphate, should be added to the embedding mass. The technique for embedding, cutting, and handling the sections is described. Sections can be cut with any ordinary paraffin microtome. It is easier to use than celloidin and considerably thinner sections can be obtained.

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